**University of Alberta**

# Examination of mtDNA polymorphisms at the Early Neolithic Shamanka II cemetery, Lake Baikal, Siberia

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Arts

Department of Anthropology

Edmonton, Alberta
Spring 2007

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395, rue Wellington
Ottawa ON K1A 0N4
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*Your file Votre référence*
*ISBN: 978-0-494-29901-2*
*Our file Notre référence*
*ISBN: 978-0-494-29901-2*

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~ For my mother and grandmother~

Your unwavering love and support mean the world to me. May I aspire to your levels of wisdom and greatness. You are truly the finest 'Hap H' ladies around…

**Abstract**

Completed as part of the Baikal Archaeology Project, this thesis examines mitochondrial deoxyribonucleic acid (*i.e.* mtDNA) polymorphisms retrieved from individuals buried at an Early Neolithic (ca. 5800–4900 BC) hunter-gatherer cemetery, Shamanka II, located on the southwestern tip of Lake Baikal, Siberia. The principal objective of this study is to compare the mtDNA polymorphisms observed at Shamanka II cemetery to the mtDNA results previously recovered from the Lokomotiv and Ust'-Ida I cemeteries. All three cemetery populations are comprised of mtDNA haplogroups characteristic of Asian origin (*i.e.* haplogroups A, C, D, F, G2a, and U5a). Results confirm that Shamanka II and Lokomotiv have similar haplogroup frequency distributions, while the haplogroup frequency distribution of the Ust'Ida I population is significantly different.

**Acknowledgements**

I would like to take the time to thank key people that contributed to my completion of this project.

Thank you to my supervisors, Dr. Weber and Dr. Bamforth. I am so appreciative to have had this unique research opportunity. Your collective wisdom, patience and guidance are truly valued and ensured my success. Dr. Weber, thank you for your generosity in funding my work and your attention in helping me improve my calibre as a graduate student. Dr. Bamforth, you are a wonderful person. I am grateful for your willingness to assist me in my research and for advising me in so many matters.

Special thanks go to the Department of Anthropology for tuition support and teaching and research assistantships. Dr. Owen Beattie and Gail Mathew deserve special mention for their assistance and encouragement. Likewise, thanks to Ben Bablitz from the Medical Laboratory Sciences for his interest and enthusiasm in my work and in my extracurricular coaching. You were very generous with your time and I appreciate all of the technical assistance that you provided. I must also thank the Faculty of Graduate Studies and Research and the Graduate Students Association for their support through grants and travel awards that enabled international conference involvement.

Members of the Baikal Archaeology Project deserve thanks. In particular, Dr. Karen Mooder, my mentor, I thank you for sharing your expertise with me and for always being supportive and approachable. I would especially like to thank Vladimir Bazaliiskii from Irkutsk State University for permitting me to work with the Shamanka II samples. Elizabeth Gustafson, my fellow bunny-suit-wearing lab-mate, we shared in some unique and challenging times. I thank you for your friendship and all of the encouragement you gave me. Andrea Hiob, I could always count on for you for anything at all – whether it be administrative in nature, or just a friendly hug… Mike Metcalf, I am grateful for all of your assistance with gathering background data on Shamanka II. Your insight and the discussions we shared helped illuminate some research ideas. Kendra Stiwich, my fellow office mate and good friend. Your advice and outlook were always solicited and most appreciated. Dearest Tanya Nomokonova, thank you for your constant friendship, for dancing and laughing with me and for sharing your time to enhance my knowledge of Baikal archaeology and Russian language skills! HeeHee…

I have had the pleasure of forming many new friendships through the duration of this project and I would like to thank these people for keeping me grounded and sane and for making life extremely fun and interesting. Patti, Mindy, Juliya, Janene, Kara, Akemi, Heather, Christine and my fiho, broomball and football folk. Thank you for being good people and for providing me with a stress outlet away from campus.

My family has been a source of unwavering support and encouragement. I love you and thank you all. And, dearest Breedley...I am most grateful for your friendship, love and relentless support despite challenging times.

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# Chapter 1
# Introduction

This thesis examines mitochondrial deoxyribonucleic acid (*i.e.* mtDNA) polymorphisms among individuals buried at an Early Neolithic (ca. 5800–4900 BC) hunter-gatherer cemetery, Shamanka II, located on the southwestern tip of Lake Baikal, Siberia. This research fits within the broader framework of the international, multidisciplinary Baikal Archaeology Project (BAP). The main objectives of BAP research have been focused on the reconstruction of life ways and the mechanism of culture change among prehistoric hunter-gatherers that inhabited the Lake Baikal region in Eastern Siberia during the Middle Holocene period.

It has been proposed that kinship lineages are represented spatially, as 'rows' of graves in Lake Baikal region cemeteries. Therefore, one aspect of this study is an independent method seeking to confirm or refute the above hypothesis. Ancient mtDNA was extracted from the preserved human skeletal remains representative of a subsample of the Shamanka II cemetery population. The likelihood of authentic nuclear DNA being recovered from human bone samples diminishes with time. It is for this reason that mtDNA is the focus of this research project and many other ancient DNA studies. MtDNA is far more abundant than nuclear DNA within a single human cell, it is maternally inherited, it undergoes little if any recombination and has a relatively rapid substitution rate (Wallace, 1999). These features of mtDNA make it possible to trace regionally-specific maternal lineages, referred to as haplogroups, through time and space. This type of information is relevant and valuable to a wide variety of researchers as it

may lend insight on matrilineally-based social practices (*e.g.,* inferred through mortuary variability, subsistence strategies), as well as human migration through time.

It must also be mentioned that archaeological excavation at Shamanka II is not yet complete and for this reason, this project must be considered as a preliminary report. All available individuals sampled in 2002 from previous Shamanka II excavations are subject to this analysis. However, with continued excavation and research, results and conclusions may change.

### 1.1. Organization of thesis

Chapter 2 provides all information relevant to this project. This includes the geographical and archaeological background of Shamanka II, as well as the region's culture history. Next, an overview of other analytical studies of the Shamanka II materials is provided. Background information on mtDNA, including its structure, organization and special features follows. Finally, this chapter also includes a review of Siberian mtDNA literature from both ancient and modern perspectives.

Chapter 3 includes a discussion of the factors that may affect DNA survival, an important consideration given that the samples under analysis in this study are prehistoric in age. This section is followed by identification and discussion of ancient DNA contamination issues and authentication criteria. Next, a thorough outline of the materials and methods undertaken for obtaining and analyzing this project's ancient mtDNA is reviewed.

In Chapter 4, the interim mtDNA results from Shamanka II are presented in conjunction with the spatial data of the cemetery. Various hypotheses are put forward in

an attempt to explain the observed mortuary variability at Shamanka II cemetery from a matrilineal perspective. This chapter also compares the results for Shamanka II to its Neolithic counterpart, Lokomotiv, as well as a Late Neolithic and Bronze Age cemetery, Ust'-Ida I.

Finally, Chapter 5 summarizes the results of this project and outlines areas to be considered for continued research and future inquiries.

### 1.2. Aims of present study

The present study has two principal research objectives: First, this research aims to compare the mtDNA polymorphisms observed at Shamanka II cemetery to the mtDNA results of a previous study. Specifically, the Lokomotiv and Ust'-Ida I cemeteries provide comparative populations for this investigation (see Mooder, 2004). Like Shamanka II, Lokomotiv is representative of the same culture group and is contemporaneous in age (ca. 5800–4900 BC) while Ust'-Ida I is representative of a Late Neolithic and Bronze Age culture group (ca. 4200–1000 BC). There is a pronounced gap or hiatus in radiocarbon dates stretching between the Early Neolithic and Late Neolithic/Bronze Age cultures (Weber *et al.*, 2002). For her dissertation research, Mooder (2004) genetically examined human remains from Lokomotiv and Ust'-Ida I cemeteries as a means of testing a biological discontinuity hypothesis, or more simply, that pre-hiatus groups were genetically different from post-hiatus groups. Mooder's (2004) results indeed revealed disparate mtDNA distributions between pre- and post-hiatus groups. Because Shamanka II and Lokomotiv are both pre-hiatus Early Neolithic

cemeteries, it is hypothesized that mtDNA results retrieved from Shamanka II will resemble those recovered from Lokomotiv and contrast those from post-hiatus Ust'-Ida I.

The second objective of this project is to examine the biological relationship (*i.e.* maternal, via mtDNA) between individuals buried together and those buried within close proximity to one another at Shamanka II cemetery. The underlying rationale of this assessment is to reveal social and/or cultural patterns that may exist in the cemetery. An understanding of the spatial patterning of maternal lineages across burial grounds can illuminate issues of social status, marriage patterns, burial customs, and differential patterns of mortality by sex.

## Chapter 2
## Background

### 2.1. Geographic context

To date, Lake Baikal has been a focal point for prehistoric hunter-gatherer research conducted by the Baikal Archaeology Project (BAP). The lake is located in the southern region of Eastern Siberia (Figure 2.1) and is the oldest and deepest lake in the world, with the largest volume of fresh water on earth (Shahgedanova, 2002).

The area surrounding Lake Baikal is divided into two regions based on geographical characteristics: the Trans-Baikal is defined as the area to the south and east of the Lake while the Cis-Baikal includes the area to the north and west of the lake. Shamanka II cemetery is located within the Cis-Baikal and is situated on the very southwestern tip of Lake Baikal, near the town of Sliudianka. The site itself is located on a promontory overlooking the lake.

The Khamar-Daban mountain range spans the southern stretches of Lake Baikal and as Bazaliiskii (2000) notes, these peaks impose limitations on the possibility of excavation and study of ancient mortuary and habitation sites along the shoreline. However, the south-southwestern shoreline offers an exception. In contrast to the mountains, this area is designated as a plains territory, which is somewhat misleading as the region contains rolling hills (Bazaliiskii, 2000a). Nevertheless, this terrain is covered in vegetation that is fed by the the Tal'ka River and Kultuk Bay. It is also the location of Shamanka II cemetery.

The climate of the Lake Baikal region is continental with warm summers, cold winters and limited precipitation. Regions of discontinuous permafrost have also been

identified in the area around Lake Baikal but not near Shamanka II; therefore, the combination of this area's cool temperatures and limited moisture favours the preservation of DNA within the osteological material recovered from excavations at Shamanka II cemetery (Weber *et al.*, 2002).

## 2.2. Archaeological context

In 1956, amateur archaeologist V.V. Svinin first discovered the Shamanka II cemetery as he noticed human bones eroding out of a promontory overlooking Lake Baikal. After his initial discovery, Svinin organized annual archaeological field trips for local schoolchildren to the lakeside (see Bazaliiskii, 2000). There is no official documentation regarding whether or not excavations were completed and there is no record of any archaeological material collected. In 1962, a teacher – student pair undertook unofficial excavations at Shamanka II. The material they collected, including human skeletal remains and associated grave goods, was stored at the Sludianka House of Young Scouts. None of the items, however, were assigned catalogue numbers and have since been unaccounted for. The first formal excavations at Shamanka II were undertaken by Kharinskii and Turkin in 1998. However, since 2000, all subsequent archaeological expeditions have been led by Bazaliiskii.

### 2.2.1. *Baikal Archaeology Project and Lake Baikal region culture history*

This study was completed as a part of the international, multidisciplinary BAP. It consists of five modules or research areas. These modules include an archaeological section, an environmental section, an ethnographic section, an osteological section, and a

genetics section. To date, the main source of research information has been obtained through the excavation and analysis of prehistoric hunter-gatherer cemeteries.

The current culture history model (Table 2.1) was developed by Weber *et al.* (2002). This model suggests that from approximately 6800 to 1000 BC (based on calibrated radiocarbon dates), the Lake Baikal area was successively inhabited by two predominant groups: the Kitoi, who date back to the Late Mesolithic and Early Neolithic, and the Serovo-Glazkovo, who date from the Late Neolithic to Bronze Age.

An interesting feature of the above model is the 800-year hiatus between the Kitoi and the Serovo groups. Radiocarbon dates from additional mortuary sites in the Lake Baikal region have led Weber *et al.* (2005) to assert that this 'gap' between the above mentioned groups may be extended to 1000 or even 1200 years in length. However, thus far, BAP research has been predominantly focused on examination of the biological and cultural characteristics of the human groups on either side of this hiatus. In general, BAP research suggests that there is significant disparity between the Kitoi and Serovo-Glazkovo cultures. These differences are noted in areas of key importance with respect to mortuary protocol, such as subsistence, diet, mobility, age and sex structure, as well as demographic trends (see Table 2.2).

From the indicators listed in Table 2.2, it is suggested that the pre-hiatus Kitoi are characterized by a pattern of relatively non-intensive use of the environment (Weber *et al.*, 2002). It is inferred that competition for resources would have been relatively low and access to resources would have been more or less unrestricted, although McKenzie (2003) states that this access may have perhaps been occasionally contested. In contrast, the post-hiatus (Serovo-Glazkovo) outline articulates a much more intensive pattern of

land use where access to critical resources may have been restricted. Therefore, it is theorized that these groups may have bridged periods of individual resource shortage by using a broader range of resources more intensively (Weber *et al.*, 2002).

Link (1999) completed a majority of the early osteological examinations of both pre- and post-hiatus Lake Baikal cemetery populations. In particular, his investigation focused on the pre-hiatus cemetery, Lokomotiv, and the post-hiatus cemetery, Ust'-Ida I. From his analyses of the Kitoi skeletal remains at Lokomotiv, Link (1999) observed features that suggested the group was in a period of demographic decline. This demographic hypothesis resulted from a comparative approach: the osteological samples from Lokomotiv cemetery were evaluated relative to samples representative of the Serovo-Glazkovo from Ust'-Ida I cemetery. Though no great pathological conditions were noted, evidence of greater periods of non-specific systemic stress were noted in the Kitoi remains, especially among juveniles. Although the etiology of this 'stress' is non-specific, it was manifested primarily as enamel hypoplasia (Link, 1999). In contrast, Link (1999) observed that Serovo-Glazkovo groups evidenced a larger number of healthy females, generally better health and longer lives, all of which contributed to a growing population. More recently, however, Lieverse (2005) revisited the Kitoi and Serovo-Glazkovo collections. After re-examination, she affirmed that, contrary to Link's previous conclusions, both pre- and post-hiatus populations revealed high levels of community health and as such, there is little reason to believe that the Kitoi population was depleted due to illness or other physiological stresses.

Nonetheless, in the Lake Baikal region, the Lokomotiv and Shamanka II cemeteries are representative of the pre-hiatus Kitoi culture. Specifically, Lokomotiv

cemetery is located in the modern city of Irkutsk, Siberia at the junction of the Angara and Irkut Rivers (Figure 2.2). Mooder (2004) relates that this cemetery was discovered accidentally in the late 1800's during construction of the Trans-Siberian Railway. Russian archaeologists from the Irkutsk State University began excavations in the 1970's and completed their work in the early 1990's. Seventy graves were excavated and 124 individuals recovered. The retrieved skeletal remains are curated in the Department of Archaeology and Ethnography at the Irkutsk State University. Vertebral skeletal samples were collected from 40 individuals in 1995 for subsequent DNA analysis to be completed at the University of Alberta Human Identification Lab for Archaeology (HILA). Furthermore, the calibrated radiocarbon dates cited by Mooder (2004) from Lokomotiv skeletal samples suggest that the cemetery was in use from approximately 6000–5000 BC. Macroscopic examination of the osteological remains excavated at Lokomotiv revealed their exceptional preservation despite being approximately 7000 years in age (Mooder, 2004).

The Shamanka II cemetery, situated on the very southwestern tip of Lake Baikal, is separated from Lokomotiv by a direct distance of ca. 80 km (Figure 2.2). To date, 70 graves have been excavated, most of which are representative of the Kitoi culture. From these graves, 120 individuals were retrieved. Excavation of the cemetery is not yet complete. Approximately 20 unexcavated graves remain.

When available, individual vertebral skeletal samples were collected and sent to the University of Alberta for DNA analysis at HILA. The preliminary calibrated radiocarbon dates from Shamanka II skeletal samples suggest that cemetery use ranged

between approximately 6000–5000 BC. This time range overlaps with the cemetery use known for Lokomotiv.

Shamanka II is the only large Kitoi cemetery to be uncovered outside of the Angara valley (note: 'small' Kitoi cemeteries do exist outside of the Angara valley, Weber, personal communication). Naturally, this discovery evokes questions of social, cultural and biological intrapopulational affinities at Shamanka II, as well as interpopulational affinities with its Early Neolithic counterpart, Lokomotiv. Preliminary genetic investigations completed by BAP researcher, Karen Mooder, have demonstrated that the Kitoi are genetically different from the post-hiatus Serovo-Glazkovo culture. However, the degree of genetic relatedness with other Kitoi groups has not yet been addressed. More information may confirm hypotheses, or illuminate factors that contributed to the alleged biocultural hiatus, as well as the fate of the Kitoi. Observed mortuary ritual, associated grave assemblages and morphological similarity in archaeology can provide clues with respect to an individual's social status and kinship relationships (Weber, 2001). Conversely, these facets of social organization cannot be directly examined without a DNA approach. Analysis of the ancient mtDNA of Kitoi individuals buried at Shamanka II provides an independent means of assessment for socio-cultural topics, such as maternal kinship relationships.

### 2.2.2 *Topographic location and spatial data*

Figure 2.3 depicts the spatial layout of the Shamanka II cemetery specifically focusing on the subsection where individuals sampled for ancient mtDNA analysis were buried. It is important to note that the site map contains both Neolithic and Bronze Age

burials. Only Neolithic graves were examined for this project. A total of 19 Neolithic graves were situated in this portion of the cemetery. Of the Neolithic graves, five were not genetically analysed for two reasons: either samples were not collected and/or available for a particular grave, or when available, samples were not the type designated for this investigation (*e.g.,* teeth). As mentioned earlier, only bone specimens were utilized for this project. MtDNA information was retrieved from 21 individuals buried within the remaining 14 graves.

Spatially, intrasite grave distribution and organization appeared to be rather random. Although, when assessed against the hillside cemetery's contour lines, as shown on the site map (Figure 2.4), four general 'rows' of graves running from east to west were apparent. In his dissertation, BAP researcher, Hugh McKenzie, cited a 'row' as "at least three closely associated parallel graves arranged in a more or less straight line" (2005:109). According to this definition, Shamanka II cemetery can indeed be regarded as being organized into four discrete rows. For instance, if one was to start at the southern end of the cemetery, Row 1 would contain graves 6, 7 and 11; Row 2 would contain graves 8, 12, 13, 14, 15, 16 and 17; next, Row 3 would contain graves 10, 18, 19 and 20; while Row 4 would contain graves 4, 21, 22, 23 and 24 (see Figure 2.4).

### 2.2.3 *Demographic data*

The demographic data for the individuals sampled from Shamanka II in 2002 were obtained through osteological examination completed by Angela Lieverse. Her assessments of individual age and sex were accomplished using standard osteological approaches.

Investigations at other Lake Baikal region cemeteries prior to work at Shamanka II permitted BAP researchers to ascertain significant differences between the pre-hiatus and post-hiatus culture groups, specifically regarding each faction's subsistence strategy and diet, as well as the dynamics of their social relations. The resultant sex ratio with respect to individuals excavated at Shamanka II fortifies the earlier notion suggested by BAP researchers that there was less gender-equity within Kitoi groups. There is a pronounced bias towards males, as they dominate the cemetery. For example, of the 21 individuals analysed for this project, 17 were male while the remaining four were female (see Chapter 4; Figure 4.1).

Despite both sexes being represented at Shamanka II, all ages were not represented in the examined sample. In fact, all of the individuals interred within the analysed section of Shamanka II cemetery could be considered 'prime-age' adults. The youngest individual is aged between 16–18 years (an age which during Neolithic times, may have been considered 'adult') while the oldest individual was aged to 45 years. Consequently both of these individuals were male. Furthermore, there were only two individuals at Shamanka II that were classified to an age of approximately 18 years and younger. Again, both of these individuals were male and they were recovered from group burials containing three and five individuals, respectively. Nonetheless, the overall mean age for males (n = 17) buried within the cemetery was 30. In contrast, females (n = 4) interred at Shamanka II were significantly younger. Their mean age was 22 (see Chapter 4; Table 4.4).

Not all sex determinations at Shamanka II were achieved through assessment of standard nonmetric osteological traits. Only 10 of the 21 individuals (48 %) were

definitively classified as male or female. Several of the remaining individuals presented ambiguous osteological sex determination features, poor preservation and/or condition, while some lacked sex-informative elements altogether. In these circumstances (n = 11; 52 %), molecular sex analyses targeting the amelogenin locus of the X and Y chromosomes were undertaken in order to obtain a more comprehensive portrait of the sex distribution in this subsample of the cemetery.

### 2.2.4 *Grave and burial characteristics*

All of the graves under analysis were oriented in a northeast-southwest direction. Actual grave pit shape was generally oval and the documented units averaged a length of 2.12 m and a width of 0.88 m. The grave depth at Shamanka II was quite variable, ranging from relatively shallow (0.80 m) to very deep (2.65 m). Among the documented graves with recorded pit depth information, the visible trend was that the majority were at least 1 m below the modern day surface, with an average depth of 1.18 m. In addition, the use of red ochre was liberal and omnipresent, as all of the examined graves at Shamanka II revealed its presence on individual skeleton(s) or covering areas of the grave itself.

Of the 14 examined graves, most revealed inhumations of single individuals (n = 8; 57 %). There were three cases of double burials, two of triple burials and a single occurrence of a multiple or group burial including five individuals (see Figure 2.4).

Of the single inhumations, all but one individual was placed in an extended supine position. This outlier was housed in Grave 12. The anatomical orientation of the individual could not be determined as few skeletal elements were represented within the

grave. However, the sex of the individual was analysed genetically and was resolved to be male.

Likewise, double burials at Shamanka II displayed some unique characteristics. Grave 14 included the remains of two individuals, a male and a female. Each was oriented in an extended supine position. However, Bazaliiskii (2000) notes that the female was buried earlier than the male, as her skeleton occurred at a greater depth with the male skeleton overlapping the superior surface of her remains. Radiocarbon dates confirm this assertion. Grave 11 also included the remains of two individuals, again one male and one female. However, in this circumstance, neither individual exhibited any type of recognizable anatomical orientation. Instead, the grave is a collection of intermingled remains that were separated by the field osteologist. The third and last double burial was Grave 24. It included the remains of two individuals, but in this case, both were male. However, one male was positioned in an extended supine position with his cranium facing northeast. The other male skeleton had no anatomical orientation and was recovered as a 'bundle' of bones located at the feet of his counterpart.

Grave 13 and Grave 21 represent the triple burials uncovered at Shamanka II cemetery. Unfortunately, only one individual out of the three recovered from Grave 13 was sampled for mtDNA characterization. This individual was an adult male. In the case of Grave 21, all individuals were adult males and were represented by nearly complete skeletons in good condition. Two of these individuals were closely associated, side by side and head to head. However, the individual closest to the northwest wall of the grave was in an extended supine position, while his counterpart was in a prone position. The third individual, also a male, was found in an extended supine position resting on top of

the two previously described individuals. The only multiple burial analysed in this study included the remains of one discrete individual and, according to Lieverse (2002), a minimum of four commingled adult individuals. Three of these individuals were genetically determined to be male.

The disorganized (*i.e.* no anatomical orientation) and disarticulated state of the human remains observed in some (n = 3; 21 %) of the 14 Shamanka II graves analysed may be the consequence of post-interment disturbance. Through these observed cases, it appears that prehistoric people routinely reopened graves after burial and removed or displaced human remains and/or artifacts (Robertson, 2006).

Four graves displayed evidence of possible unusual treatment of the head. Graves 12, 16, 18, and 23 (Table 2.3) had missing skulls, a trend which is of an intriguing nature. In particular, Graves 16 and 18 stand out because while the skull was missing, the postcranial skeleton was relatively well-preserved. In his dissertation, McKenzie (2006) points out burials without heads as a documented feature of Kitoi mortuary practices witnessed at Shamanka II. He also adds the presence of "stray bones" (see Bazaliiskii, 2003) as an additional distinguishing trait of Kitoi burials. Evidence of this practice is recorded in two graves (8 and 19) where a "foreign human mandible" and a "foreign human vertebra," respectively were recovered (Bazaliiskii, 2000a & 2000b).

### 2.2.5 *Grave inclusions*

Comprehensive analysis of the grave inclusions from Shamanka II has yet to be completed. However, the general distribution of grave goods recovered from the 14 analysed graves for this project varied in regard to number and kind of interred objects, as

well as their placement within each respective grave. In fact, some of these Shamanka II graves revealed an abundance and variety of artifactual materials, while others had none (see Bazaliiskii, 2000a and 2000b for more detailed information).

There was a general similarity of artifact types seen in the graves excavated at Shamanka II, in comparison to other Lake Baikal region cemeteries of similar age, such as Lokomotiv (Bazaliiskii, 2003). However, one particular grave inclusion was a first-time discovery. This object was described as a "bent bone holder for insert tools" or, more clearly, a composite bone handle with a manufactured groove for inserting lithic blades. Other retrieved items included various animal tooth pendants, harpoons of antler and bone, nephrite adzes, calcite discs, slate, argillite, and chert flakes/blanks, lithic projectile points, bone needles, carved antler effigies of seal and moose, and the distinctive Kitoi composite fishhooks.

Most of the above-mentioned objects were uncovered at the burial level of a grave, indicating that there may be a meaningful association with the interment. The presence of rodent and carnivore teeth and mandibles in Kitoi graves at Shamanka II was also widespread and according to Bazaliiskii (2003), these items are not uncommon grave inclusions. Bear crania and mandible remains were recovered in half of the analysed graves (n = 7; 50 %) at Shamanka II. According to Oakes and Riewe (1998), the bear is an animal of great significance for the indigenous peoples of Siberia and among the Khanty, Mansi, Sel'kup and Kets, who reside in the taiga in Western Siberia, feasts and games are held when a bear is killed. Moreover, it is a traditional practice among the aforementioned groups that the head of a dead bear be placed in a position of prominence inside the cabin of the person who killed it. Perhaps this custom was retained from as

early as the Neolithic- and individuals with bear crania, mandibles or teeth at Shamanka II indicate a successful hunter. However, the significance of the bear varies ethnographically. For instance, among the Nenets who occupy the arctic tundra and forest-taiga region that extends from the Berents Sea eastward to the Yenisey River, the "brown bear is considered the master of the forest, embodying justice on earth" (Oakes and Riewe, 1998:9). In stark contrast to the Nenets, among the Evenki bears are seen as animals of the lower or dark world (Oakes and Riewe, 1998).

Additionally, artifacts were frequently found around the head and thoracic region of an interred individual. With respect to calcite discs and animal tooth pendants, their association with these regions of the body may have been related to a headdress or necklace, or something of a similar fashion. Nearly every analysed grave (n = 13; 93 %) at Shamanka II revealed the presence of some type of pendant – even in circumstances where a mere two grave inclusions (both boar canine pendants) were recovered (*e.g.*, Grave 24).

### 2.2.6 *Overview of analytical studies of the Shamanka II materials*

All 21 individuals analysed in this study were successfully radiocarbon dated (Isotrace Laboratories, Toronto). The assigned dates are recorded in Table 2.3 and all fall within the Early Neolithic period that has been defined by BAP researchers. These dates span from 5915 – 4840 years BC (calibrated) with a time range therefore equal to 1075 years.

BAP post-doctoral researcher Caroline Haverkort has been examining strontium stable isotope signatures from individuals buried at Shamanka II, as well as other

prehistoric cemeteries within the Lake Baikal region (Weber *et al.*, 2003). The underlying premise of this type of inquiry relies on the fact that strontium is incorporated into the human bone and dental tissues through consumption of food and water endemic to a particular geological region. The inorganic portion of human bones undergoes remodeling every 7 – 10 years in contrast to tooth enamel, which is essentially inactive after its formation in early childhood (Lowenstam and Weiner, 1989 cited in Weber *et al.*, 2003). Therefore, comparison of the stable strontium isotope ratios between bone and tooth can reflect differences (if they exist) revealing where an individual grew up and where he/she died. Overall, when successful, this method would permit insights into prehistoric mobility patterns. However, this area of research is still a work in progress. Currently, strontium isotope signatures sampled from femora at Shamanka II are available.

### 2.3. Mitochondrial DNA (mtDNA) background

Kaestle and Horsburg (2002) state that within the context of aDNA studies, there are two main DNA sources: organellar and nuclear. The vast majority of an individual's total genomic DNA is present in the nucleus of a cell. Human cells have a single nucleus therefore making retrieval of nuclear DNA from ancient remains a frequently unsuccessful task (Kaestle and Horsburg, 2002). In contrast, however, hundreds of mitochondria are present in each human cell and each mitochondrion contains numerous copies of the small circular DNA molecule. Thus, the likelihood of successful retrieval of mitochondrial DNA (*i.e.* mtDNA) from ancient remains increases proportionately. For

this reason, aDNA studies to date have generally concentrated on recovering this type of DNA.

### 2.3.1 *Structure and organization of mtDNA*

The mtDNA molecule is a closed circular structure capable of independent replication (Anderson *et al.*, 1981). It codes for 22 distinct transfer RNAs and for 13 protein genes, which are mostly involved in electron transport and the oxidative phosphorylation (OXPHOS) pathway- the energy producing system of the cell located within the mitochondrial inner membrane. This system is made up and controlled by the products of genes encoded in the nuclear DNA and mtDNA. The majority of proteins of OXPHOS enzyme complexes are encoded in the cell nucleus and are transported to mitochondria from cytosol. Most of the genes are transcribed from guanine-rich heavy strand (H-strand) of mtDNA. Only the sixth subunit of complex I and eight tRNAs are transcribed from the cytosine-rich light strand (L-strand) (Wallace *et al.*, 1999).

The organization of most mammalian mtDNA genome(s) is extremely conservative and economical (Saccone *et al.*, 1999). In particular, human mtDNA practically lacks noncoding regions altogether while all of the coding sequences are contiguous (Anderson *et al.*, 1981). The largest noncoding region of the mtDNA genome is referred to as the D-loop or control region. Specifically, it is a 1.1 kilobase (kb) segment that contains the control elements for replication and transcription of mtDNA (Lightowlers *et al.*, 1997).

The 16,569 base-pair genome is maternally inherited, allowing one to trace maternal lineages through time. MtDNA undergoes little, if any recombination and has a

high substitution rate. In particular, over subsequent generations of inheritance, neutral mutations occur and accumulate in an individual's mtDNA, creating distinct polymorphisms which, in general, are regionally specific. These features of mtDNA are discussed further below and more importantly, allow geographic-specific maternal lineages, known as haplogroups, to be traced through time and space. For this reason, researchers (*e.g.*, Kaestle and Horsburg, 2002; Mooder, 2004) emphasize the value of mtDNA as a tool for anthropological investigations, especially when seeking to reveal the origin and migration of maternal lineages over time.

### 2.3.2 *Special features of mtDNA*

Compared with nuclear genes and nuclear DNA in general, mtDNA has a few special characteristics that make it a useful marker for phylogenetic and phylogeographic studies. As mentioned above, these features are: maternal inheritance, lack of recombination, homoplasmy and a relatively high mutation rate.

The maternal mode of inheritance and the lack of recombination in mtDNA (Merriwether *et al.*, 1991) offer potential to track individual genealogies and their evolution through the genetic history of human populations. Due to this lack of recombination, mtDNA acts as a single locus. The effective population size of the mitochondrial genome is only one fourth that of the autosomal loci. The influence of genetic drift thus makes the mitochondrial genome more sensitive to random fluctuations of allele frequencies than that for the autosomal loci.

Various tissues of the same individual usually share only one type of mtDNA; this condition is referred to as homoplasmy. When a mutation arises, there may be complete

switching to the new mtDNA variant within a single generation (Poulton *et al.*, 1998). If the switching is incomplete then two or more types of mtDNA can be observed in a cell. This state is called heteroplasmy.

On average, mtDNA accumulates mutations more than 10 times faster than does the nuclear genome (Ingman and Gyllensten, 2001). This phenomenon leads to high-level of polymorphism within populations. A high mutation rate of mtDNA is associated with several properties of mtDNA, different from those of the nuclear genome and with the peculiarities of processes taking place in the mitochondrial OXPHOS pathway. Firstly, mtDNA lacks protective proteins like histons. Secondly, mtDNA is exposed to oxidative damage by reactive oxygen radicals, the by-products of OXPHOS. Thirdly, it has been suggested that the reparation system of mtDNA is not as effective as that in the nucleus (Bogenhagen, 1999), although many elements of the mtDNA repair system have been shown to exist in animal mitochondria (Lightowlers *et al.*, 1997).

Overall, the fast evolving mtDNA provides more information about recent events in evolution than does a stretch of DNA in the nuclear genome (Lightowlers *et al.*, 1997).

**2.4. Review of mtDNA research in Siberian anthropology**

Pääbo and colleagues (2004) propose that the study of ancient DNA has "the allure of time travel." Indeed, an investigation where authentic ancient DNA (aDNA) is successfully retrieved from human bone samples allows researchers to analyse aspects of the biological history of prehistoric individuals and populations. When complementary evidence is available, such as archaeological data, the results can be very helpful in reconstructing past lifeways.

This section intends to review current and available literature pertinent to mitochondrial DNA-based studies of Siberian (*i.e.* 'East Asian') populations; this includes both ancient and modern human mtDNA investigations. Together, these temporally discrete perspectives are of importance because such data can be used to reconstruct ancestor-descendent relationships between populations, and to discern patterns of interrelatedness between ancient groups. This information can also be used to reveal the origin and migration of past populations through biological comparisons of ancient and modern Siberian population mtDNAs.

### 2.4.1. *Ancient mtDNA*

O'Rourke and colleagues (2000) published a review that examined the efficacy of aDNA studies within the field of physical anthropology. They assessed factors that inhibit aDNA preservation as well as a variety of methods for the extraction and amplification of DNA from ancient human samples. Specifically, topics included: biochemistry of aDNA; extraction methods; sources of DNA; amplification methods; authenticity of aDNA; and applications of aDNA research in physical anthropology presented by continent. Overall, studies with aDNA are deemed pertinent to anthropology, especially in regards to the subdisciplines of archaeology and physical anthropology. O'Rourke *et al.* (2000) cite that, in general, ancient human mtDNA investigations can be used to address questions in human population history and migration, as well as human evolution. Future research prospects incorporating aDNA analysis with anthropology can result in refinement and greater understanding of primate and human taxonomic and evolutionary studies.

Great similarity exists between the work of O'Rourke *et al.* (2000) and the subsequent "aDNA and Anthropology" summary by Kaestle and Horsburgh in 2002. Like the former, Kaestle and Horsburgh (2002) reiterate DNA background, as well as the methods and applications of aDNA studies from a physical anthropology perspective. In contrast to O'Rourke *et al.* (2000), these investigators present a more applied approach for aDNA studies and consider anthropological applications for aDNA work from an individual, a family, a local, a population, and a species level. For instance, an 'individual' level focus may examine topics such as sex determination; a 'family' focus may outline pedigrees to trace relationships and rare mutations; a 'local' focus may examine inheritance and residence patterns *(e.g.* endogamy/exogamy; matrilineality/patrilineality) by comparisons of estimates of levels of variation within mtDNA, Y chromosome and autosomal DNA; a 'population' focus may investigate the movement and/or migration of peoples, as well as intra/intersite continuity or replacement; and finally, a 'species'level focus may assess the relationship between humans and other hominids.

Additionally, Kaestle and Horsburg (2002) also consider nonhuman sources of aDNA and their research applications. Animals recovered from archaeological sites can hold valuable insight with respect to past human behaviour. For instance, cultural practices, seasonal population movement, diet, migration, environmental reconstruction and infectious disease are all subjects that may be inferred through the presence and analysis of animal remains. When morphological indicators cannot identify these animals, an aDNA approach can be undertaken. In particular, a genetic research

approach can reveal information such as species identification and domestication practices.

Ricaut and others (2004) apply some of the approaches discussed above and link genetic analysis with physical anthropological and archaeological studies. In general, many researchers employ a genetic approach to reveal biological population affinities and then apply this information to deduce other important population elements – such as movement and migration over time. Ricaut *et al.* (2004) undertook an aDNA approach in their analysis of two skeletons belonging to the Scytho-Siberian population, an Indo-European group that lived in the Eurasian steppe zone of Central Asia ca. 2,500 BP. They were seeking to determine the genetic relationships between the two subjects as well as to search for their genetic population affiliations. In particular, their approach included analysis of autosomal short tandem repeats (STRs) and mtDNA HV1 sequencing. Their results revealed genetic differences between these two individuals; they were not closely related. Furthermore, haplogroup analysis confirmed that one of the individuals belonged to F2a, while the other was classified to D. These researchers emphasize that both of these haplogroups are characteristic of Asian populations. Ricaut *et al.* (2004) provide two hypotheses to explain their results; however, with only two samples, the validity of their results should remain in question until more data are obtained. Overall, however, through their genetic investigation, these researchers concluded that these two ancient individuals were not close relatives, but are both linked to Asian populations.

In addition, Ricaut and colleagues (2005) were involved in a subsequent genetic investigation. An archaeological expedition in 1980 saw the discovery of a single frozen

Neolithic grave (ca. 3600 BP) in the Sakha Republic of northeastern Siberia. The anthropological investigation determined that interred individual was a young female (20–25 years old) and presented skeletal indications (*i.e.* skull traits) of Asian ancestry (Gokhman and Tomtosovoy, 1983 after Ricaut *et al.,* 2005). Moreover, the archaeological evidence deemed that this woman could not be affiliated with known recent or ancient Siberian ethnic groups (Kistenev, 1992 after Ricaut et al., 2005). It is for this reason that Ricaut and researchers (2005) undertook a genetic approach in hopes of revealing this individual's population affiliation. A bone sample collected from the femur of the ancient skeleton was prepared and its aDNA was extracted. MtDNA polymorphism analysis was completed on the HVI region of the molecule. Results of this analysis led to a haplogroup C classification. This haplogroup is widespread in modern Asian populations and most notably in east and south Siberian and Asian populations. Haplogroup C is also considered to be one of the four founding Native American haplogroups and is hence widespread among Native American populations. Therefore, Ricaut *et al.* (2005) emphasize that this shared haplogroup in east Siberian/Asian and Native American populations could be due to either convergence or common ancestry. Additionally, autosomal STRs were examined. Ricaut and others (2005) employed an assignment method of analysis that links populations based on shared allelic frequencies of the STR loci. Overall, their results were interesting in that the probability of observing an individual with the ancient STR profile was 10 times more likely to occur in Native Alaskan, Native West Canadian and East Asian populations in contrast to other Native American populations located to the south and European populations. The results reported by these researchers present an ancient

nuclear profile and mtDNA sequence of a Neolithic northern Siberian individual. More importantly, their results are indicative of a maternal lineage and nuclear DNA link to both east Siberian/Asian and Native American populations.

Egyin Gol is a cemetery in northern Mongolia that was used from the 3rd century BC to the 2nd century AD. It is connected with the Xiongnu period and is therefore of interest to a number of researchers. The research focus of Keyser-Tracqui *et al.* (2003) was to discern biological kinship within the necropolis population; therefore, a genetic approach was employed to complete this examination. They state that their approach is important not only with respect to understanding genetic relationships within and between burial sites but also because it assists in the general reconstruction and organization of funereal places. Furthermore, in regards to mobility, this type of approach enables a connection between the genetic affinities uncovered at Egyin Gol with other [ancient and modern] geographically separate populations.

More than 80 % (re: 84 out of 103) of the graves at the Egyin Gol site were undisturbed. Consequently, Keyser-Tracqui and colleagues (2003) highlight that most of the human skeletal material sampled was in an excellent state of preservation, providing them ideal specimens for aDNA analyses. The researchers employed three types of molecular markers (STR, Y-chromosome and mtDNA) into their research design and thus generated biparental, paternal and maternal DNA profiles for over 60 individuals. The results of the genetic profiling were evaluated in the context of the cemetery's spatial layout to assess whether burials were patterned to reflect kinship.

The cemetery was partitioned into four sectors. The earliest sector was found to have the fewest number of related individuals; only a single parent-child set of burials

was observed there. Moreover, it was in this part of the cemetery that the sole high status burial was noted. The individual was male and did not share a genetic link with any other individual in the cemetery. A collection of double interments surrounded this burial and comprised its periphery. Interestingly, Keyser-Tracqui and others (2003) advise that this patterning was not uncommon for high status burials from this era and cite ethnographic and archaeological evidence in support of their claim (Francfort *et al.*, 2000). In the remaining cemetery sectors, numerous familial relationships were observed between people buried proximal to each other. These relationships included parent-child and sibling pairs. For instance, a distinct cluster of male burials was identified. This grouping was found to share a single Y-chromosomal lineage and a single mtDNA lineage. The STR profiles generated from these individuals, however, suggested that membership in this group extended beyond father-son or brother-brother pairs. Overall, close relationships between several specimens were established and these results provide additional background information with respect to social organization and funerary practices among the Xiongnu people.

In Mooder's (2004) review of the Egyin Gol article, she notes that, unfortunately, the authors did not attempt to compare biological profiles between individuals within double interments to understand if kinship played a role in this type of mortuary behaviour. She comments that overall, the paper was largely qualitative and would have been greatly enhanced if greater consideration had been paid to associations between genetic and archaeological data.

The Egyin Gol cemetery in northern Mongolia is of interest to Mooder and other BAP researchers due to its proximity to Lake Baikal, as well as its genetic-based

approach to population and kinship analysis. Mooder *et al.* (2005) conducted an investigation in an attempt to understand whether observed mortuary variability at a large Neolithic cemetery in the Lake Baikal region of Siberia, Lokomotiv, was used to signify differential biological affinity with respect to the individuals buried there. In order to assess this hypothesis, 37 individuals from the Lokomotiv cemetery were examined for mtDNA polymorphism. This assay allowed for determination of mtDNA haplogroups and their respective distributions. The results from Lokomotiv inform that matrilineal affinities were not an overt factor in the spatial organisation of the cemetery. Despite this, however, Mooder *et al.* (2005) reveal other evidence that suggests that matrilineal affinities may have influenced the type of grave an individual was interred in, as well as the type of mortuary treatment an individual received. Though the sample size investigated was small relative to the entire cemetery population, it was proposed that continued research and study of this data, as well as other pertinent data from contemporaneous Lake Baikal region cemetery sites will enhance understanding of prehistoric Siberian social complexities.

Likewise, Lalueza-Fox and colleagues (2004) undertook an ancient mtDNA approach to help clarify the debate regarding Western and Eastern genetic influences in Central Asia from the 15$^{th}$ century B.C. to the 5$^{th}$ century A.D. In addition to archaeological evidence from various prehistoric sites in Kazakhstan, they analysed mtDNA sequences and haplogroups. Their goal was to help unravel some of the early steppe migrations through the study of heterogeneous populations of Central Asia. Thirty-six tooth samples (each representative of discrete individuals) were collected from various Kazakh archaeological sites of various ages and their respective mtDNA

extracted. Twenty-nine of these samples yielded DNA and were thus sequenced (HVI), followed by haplogroup determination. Most of the retrieved sequences (n = 21; 78 %) belonged to the European or west Eurasian mtDNA haplogroups (HV, H, T, I, U, and W). The east Eurasian haplogroups (22 % of the sequences) were represented by sequences belonging to haplogroups M, G and A. More interestingly, haplogroups present in modern Kazakhs (such as B, F, C, Z, D, R, J and Y; data cited from Comas *et al.* 2004) were not observed in the prehistoric Kazakhs of this study. Overall, these results have important implications with respect to prehistoric movement and migration through Central Asia. Lalueza-Fox and colleagues (2004) close their article with the point that this study demonstrates the usefulness of aDNA as an interpretive tool for hetergeneous populations and reconstruction of past migrations.

### 2.4.2. *Modern mtDNA*

Kivisild and researchers (2002) were interested in determining the 'phylogenetic backbone of the East Asian mtDNA tree.' In addition to numerous East Asian studies cited in their work, Kivisild *et al.* (2002) collected data by sampling blood of contemporary Han individuals from southern China and then sequenced both the coding and control regions for each respective sample. Their analyses led to results that support the idea of a regionally specific East Asian mtDNA pool. As well, the feature that these mtDNA polymorphisms all stem from one of the two superhaplogroups, M and N, was reinforced. In particular, superhaplogroup M includes haplogroups C, D, E, G and Z. Conversely, superhaplogroup N is comprised of haplogroups A, B, F and Y. Their studies revealed additional haplogroups ('subhaplogroups') and these are subsequently

described. In conclusion, these researchers emphasize that through subdividing the hierarchy of the mtDNA tree into branches, boughs and twigs, settlement processes of a particular region in a particular time can be discerned.

Kong and others (2003) further investigated the phylogenetic relationships of East Asian mtDNA haplogroups. Previous research had been geared towards resolving these relationships; however, the most basal haplogroups and minor subhaplogroups relative to East Asian mtDNA had not been fully characterized in some cases. Therefore, the goal of the investigation by Kong *et al.* (2003) was to provide a more comprehensive and detailed view of the mtDNA haplogroups evident in East Asia, as well as their phylogenetic relationship. In order to achieve this, these researchers gathered more than 2000 individual samples from across China. From complete sequencing of these samples, 48 mtDNAs were identified and their phylogenetic relationships reassessed through comparison to the classifications available in previous literature. The results of their work confirmed the previous definitions of some haplogroups that were made by researchers (e.g. Kivisild *et al.*, 2002). These haplogroups remained unchanged. However, some haplogroups were broadened and their phylogenetic status reinforced. Kong *et al.* (2003) conclude their work by reinforcing the applicability of detailed phylogenetic study to the study of mtDNA-related disorders. The identification of polymorphisms is important in that pathogenic and/or disorder-associated mutations can be distinguished from haplogroup-defining mutations.

Schurr (1999, 2000, 2003, 2004a, and 2004b) has completed numerous mtDNA analyses with respect to indigenous Siberian groups, including the Koryaks, Itel'men, Altaians, and Nivkhi. Mongolian groups were also incorporated into these investigations.

This area of research is of interest primarily due to genetic evidence, which formulated one hypothesis for the peopling of the New World: that founding North American populations stem from migration and colonization events of indigenous Siberian populations. In fact, the overwhelming evidence linking Native Siberians to Native Americans manifests itself genetically in that these two groups share four primary maternal haplogroups (A, B, C, and D; Schurr, 2003). In a majority of his work, Schurr *et al.* (1999, 2000, 2003, and 2004b) compares mtDNA variation in Native Siberians and Native Americans, and assesses mtDNA haplogroup origins in Asia and Siberia. In general, most of the mtDNA datasets that Schurr *et al.* (2000, 2003, and 2004b) have analysed in contemporary indigenous Siberian groups suggest that these groups have genetic ties and may have played a role in the dispersal of ancestral Native American populations. Schurr (2003) also comments on the origins and affinities of ancient populations from the Lake Baikal region of Siberia. Overall, almost all of his work echoes the same concluding message: he advocates that continued research from both modern and ancient mtDNA perspectives of Siberian sources are promising, as there is still much insight to be gained with respect to population history.

The work of Neel and colleagues (1994) refutes the commonly accepted theory that the indigenous peoples of the New World are derivatives from ethnic groups of eastern Siberia. Their reasoning lies in the fact that a majority of eastern Siberian ethnic groups generally lack one of the primary 'founding' haplogroups well represented in Amerindian populations, haplogroup B. Alternatively, Neel and others (1994) put forward a hypothesis stating that the most proximal Asian ancestors of all Amerindians share a common origin with the indigenous people of the general region now designated

as the Siberian Far East. In order to test this hypothesis, these researchers undertook virological and genetic analyses of 473 ethnic Siberian subjects, including Siberian Eskimos, Chukchis, Chavchuven Koryaks, Nymylan Koryaks, Nganasans, Yukaghirs, Evens, Udegeys, Sel'kups, and Nivkhi. Indeed, the results of Neel *et al.*'s (1994) analyses revealed that 11 of 38 Amerindian tribes possessed T-cell lymphotrophic virus type II. More importantly, this virus was not present in any of the 10 eastern Siberia ethnic groups sampled. Conversely, Neel and researchers (1994) found that this virus has been reported in indigenous populations of Mongolia; as well, haplogroup B is represented in this region. Therefore, based on these facts- in combination with their own findings, these investigators propose that the ancestors of the first migrants to the New World were not derived from north and central Siberia, but instead from populations to the south- including regions of Mongolia, Manchuria and/or southeastern Siberia.

Kolman *et al.* (1996) reinforce the above findings. In 1996, these researchers completed a similar investigation- attempting to reveal biological affinities between Old and New World populations with respect to origin of founding ancestors. Indeed, based on their own genetic analyses of 103 Mongolians, these researchers came to a similar conclusion as Neel *et al.* (1994). Like the latter, they propose that indigenous populations in east Central Asia, including Mongolian, Tibetan and central Chinese populations are the only groups that carry all four haplogroups and exhibit the highest percentage (48%) of New World haplogroups; therefore, they appear to be the strongest candidates for New World founder populations.

Additionally, Fedorova and colleagues (2003) investigated the mtDNA lineages present in Yakuts, an indigenous population residing in East Central Siberia. Although

these researchers never explicitly state a hypothesis, it is inferred that they are interested in the origin of this ethnic population. One hundred and ninety-one samples were collected and analysed from Yakut individuals, all of which were maternally unrelated. The classification results of Fedorova and others (2003) from collected samples led to a total of 14 haplogroups and 67 haplotypes. Most of these haplotypes (92 %) belonged to haplogroups A, B, C, D, F, G, M, and Y. These types are specific to east Eurasian ethnic groups. The remaining haplogroups (8 %) were specific to west Eurasian ethnic groups, including haplotypes H, HVI, J, T, U, and W. Furthermore, the highest diversity was observed in haplogroups C and D, which comprised 44 % and 30 % of the haplotypes, respectively. With these diversity calculations, as well as the dominance of the east Eurasian haplogroups among the Yakut, the authors are content in concluding a South Central origin for this group.

The Mansi indigenous group resides in western Siberia near the Ural Mountains. They are a population of biological interest due mostly to the location of their settlements (Derbeneva *et al.*, 2002). This feature has made the Mansi a focal point of research as they are often regarded to be the proverbial 'link' between distinctly west Eurasian and east Eurasian lineages. Derbeneva and colleagues (2002) surveyed the mtDNA of 98 contemporary Mansi individuals hoping to reveal genetic clues of their history. The results of this analysis showed 63 % of the entire Mansi mtDNA dataset classified into western Eurasian lineages (*e.g.* haplogroups UK, TJ, and HV). The remaining 37 % encompassed eastern Eurasian lineages *(e.g.* haplogroups A, C, D, F, G, and M). Derbeneva and others (2002) suggest that these results indicate aboriginal residents living directly east of the Ural Mountains may include the remaining ancestors of an early

Upper Paleolithic population expansion from the Middle East and/or southeastern Europe. Furthermore, these researchers speculate that the presence of eastern Eurasian mtDNA lineages in the Mansi introduces new hypotheses with respect to ancient population movement, migration and expansion. From this new molecular information, Derbeneva and researchers (2002) hypothesize that early Eurasians may have been comprised of a range of superhaplogroup M and N lineages that subsequently became geographically distributed. Therefore, this proposed Paleolithic expansion may have reached the Mansi-region of northwestern Siberia before these superhaplogroups diverged into western and eastern human groups. Alternatively, these results could also be explained by different episodes for respective east and west admixture. In order to rule out this possibility, it would be useful to calculate the timing of the purported divergence by applying the principles of the 'molecular clock.' Though this method is not perfect, it provides potential to reveal whether one influence is more antique than the other. Indeed, if one influence is considerably older than the other, the proposed conclusion would have to be re-evaluated.

### 2.4.3. *Summary*

Generally, Siberian mtDNA is classified as 'East Asian' due to the region's geographic location. However, it is also common to see Siberian mtDNAs referred to as 'Eurasian.' Nevertheless, the phylogeny of East Asian mtDNA haplogroups had not been well examined until recently. This scientific focus for discerning the phylogenetic status of the regionally specific East Asian haplogroups is a helpful undertaking that allows researchers greater genetic resolution in the study of ancient populations.

As mentioned earlier, Kivisild *et al.* (2002) state that East Asian mtDNAs belong to two 'superhaplogroups.' For the purpose of classification, these two superhaplogroups, M and N, are described as the 'trunks' of the analogous phylogenetic tree. The M trunk encompasses the known Asian-specific haplogroups, including C, D, E, G and Z; while the N trunk includes haplogroups branches A, B, F and Y (Figure 2.5; Kivisild *et al.*, 2002).

Schurr (2003) declares that haplogroups A through D actually represent the minority of mtDNA lineages in modern Siberian and East Asian populations. In fact, nearly all contemporary Siberian groups lack haplogroup B. Furthermore, haplogroup A is generally found in low frequencies in Siberian populations, while the frequency and distribution of haplogroups C and D rise significantly (Schurr, 2003). Haplogroup F is also present in modern Siberian populations. Mooder (2004) found this haplogroup to be the most common mtDNA lineage present among the Early Neolithic (6000–5000 BC) Lokomotiv cemetery population. The complete haplogroup classification results from this cemetery are shown in Table 2.4

Schurr (2003) points out that it is interesting that F is the predominant haplogroup retrieved from the Lokomotiv cemetery population. His interest stems from the fact that this haplogroup, though not absent, is sparsely seen in most modern Siberian populations. Mooder (2004) addressed this issue of biological affinity through the completion of a principal component map (PCM) based on the statistical R matrix. The populations investigated in the analyses were two prehistoric Lake Baikal region cemetery populations and fourteen modern East Asian populations. The premise of a biological affinities analysis via PCM is that, if maternal Kitoi descendants remained in the Baikal

area, a similar distribution of mtDNA haplogroup proportions should be recognized amongst ancient and modern populations. However, results where no association is found would indicate that Kitoi descendents left the Baikal area. Mooder's (2004) PCM findings revealed an observable clustering between two groups, indicating a biological relationship. These groups were the Kitoi of Lokomotiv cemetery and the modern day Kets living in the Yenisei river basin of Siberia. Therefore, this discovery yielded an important biological link between the prehistoric Kitoi population buried at Lokomotiv and the modern Siberian indigenous group, the Kets.

It is also significant to mention that Schurr (2003) and Mooder (2004) both record that modern and ancient populations have some frequency of 'other' mtDNA. This designation means that some individuals possessed 'other' mtDNA belonging to a haplogroup that is not A – D; typically, G, Y, and Z comprise these 'other' haplogroups included in this all-encompassing category.

The literature reviewed in this section reveals the generally greater focus that is placed on modern mtDNA work among Siberian populations. It may also be the obvious result of not only the arduous nature of aDNA retrieval, but of the fact that prehistoric human remains are hard to come by. Overall, aDNA analyses have a valuable place in the array of anthropological research. In accordance with Kaestle and Horsburg (2002), such studies must not be undertaken merely to demonstrate that surviving DNA is present in organic remains; but rather, it should be endeavoured to answer anthropological questions regarding the past as well as the present.

Another trend apparent from the literature reviewed in this chapter is that aDNA studies are no longer focused solely on mtDNA. Instead, a more inclusive or integrative

approach, where Y chromosome and nuclear STRs, is becoming routine. Future avenues of aDNA research within an anthropological framework may attempt to investigate alternative perspectives, such as topics of socio-cultural relevance. For instance, though generally unseen, molecular data obtained from ancient human remains can elucidate patterns of social structure. Genetic analyses allow for sexing of human remains (particularly useful with fragmentary or subadult remains), as well as the development of an understanding of the spatial patterning of maternal and paternal lineages across burial grounds. From such data, light can be shed on issues of social status, marriage patterns, burial customs, and differential patterns of mortality by sex- in addition to the origin and migration insight traditionally revealed through ancient mtDNA studies.

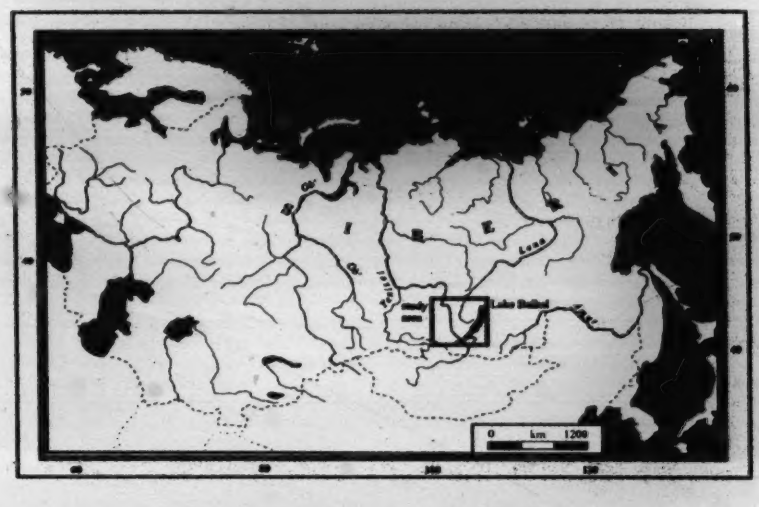


**Figure 2.1** Lake Baikal region, Siberia

**Figure 2.2** Kitoi cemetery sites in Lake Baikal region

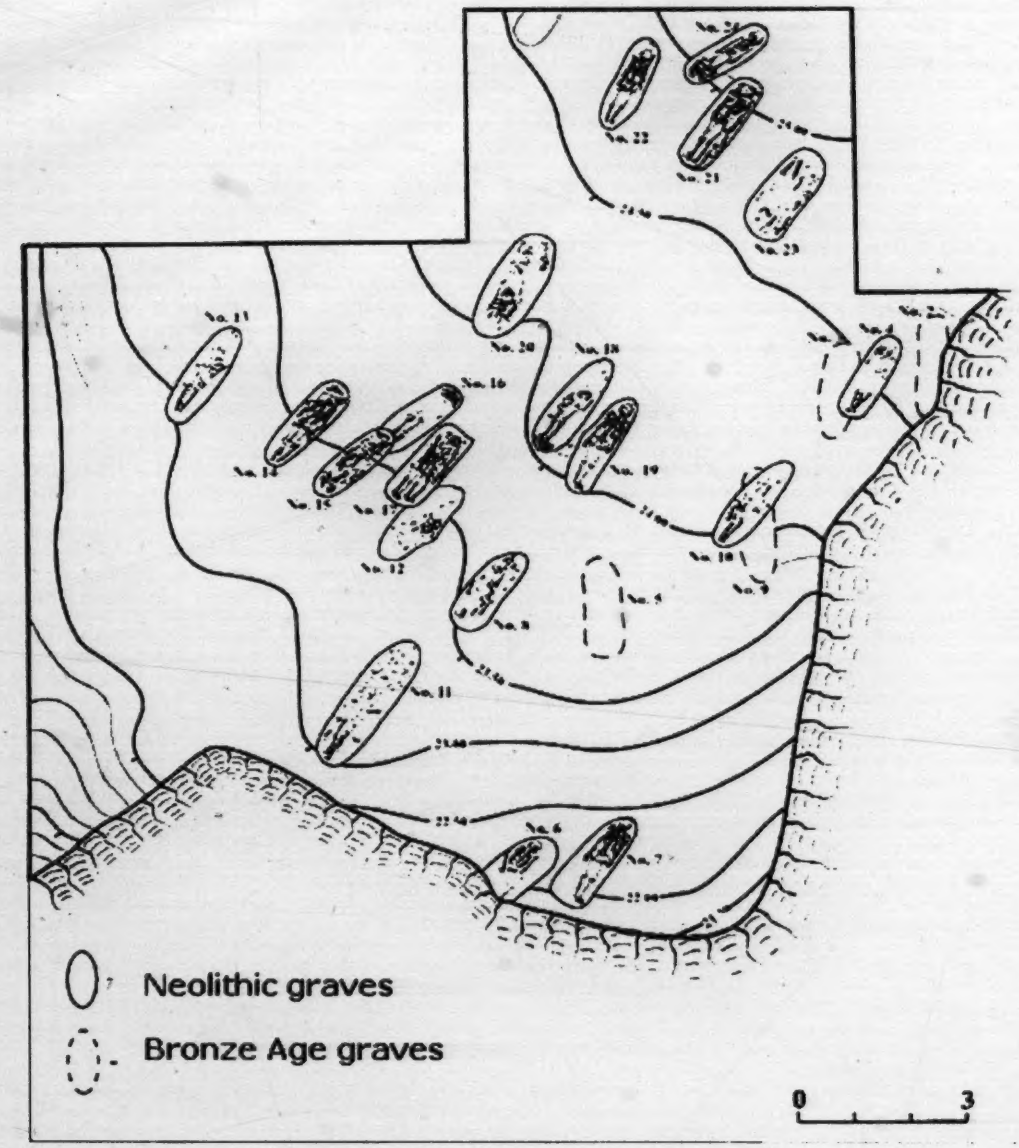


**Figure 2.3** Shamanka II cemetery plan

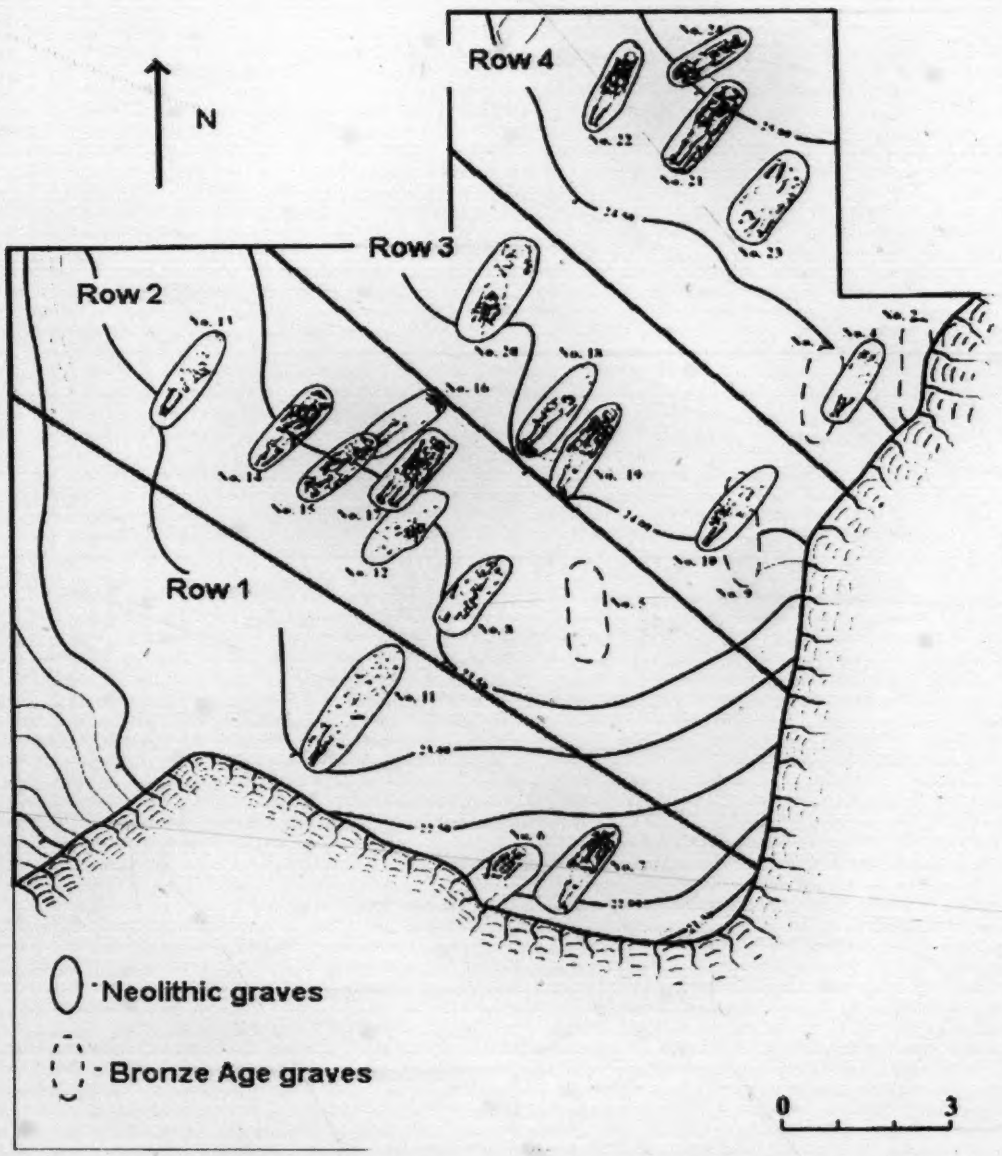


**Figure 2.4** Generalized 'rows' of graves at Shamanka II

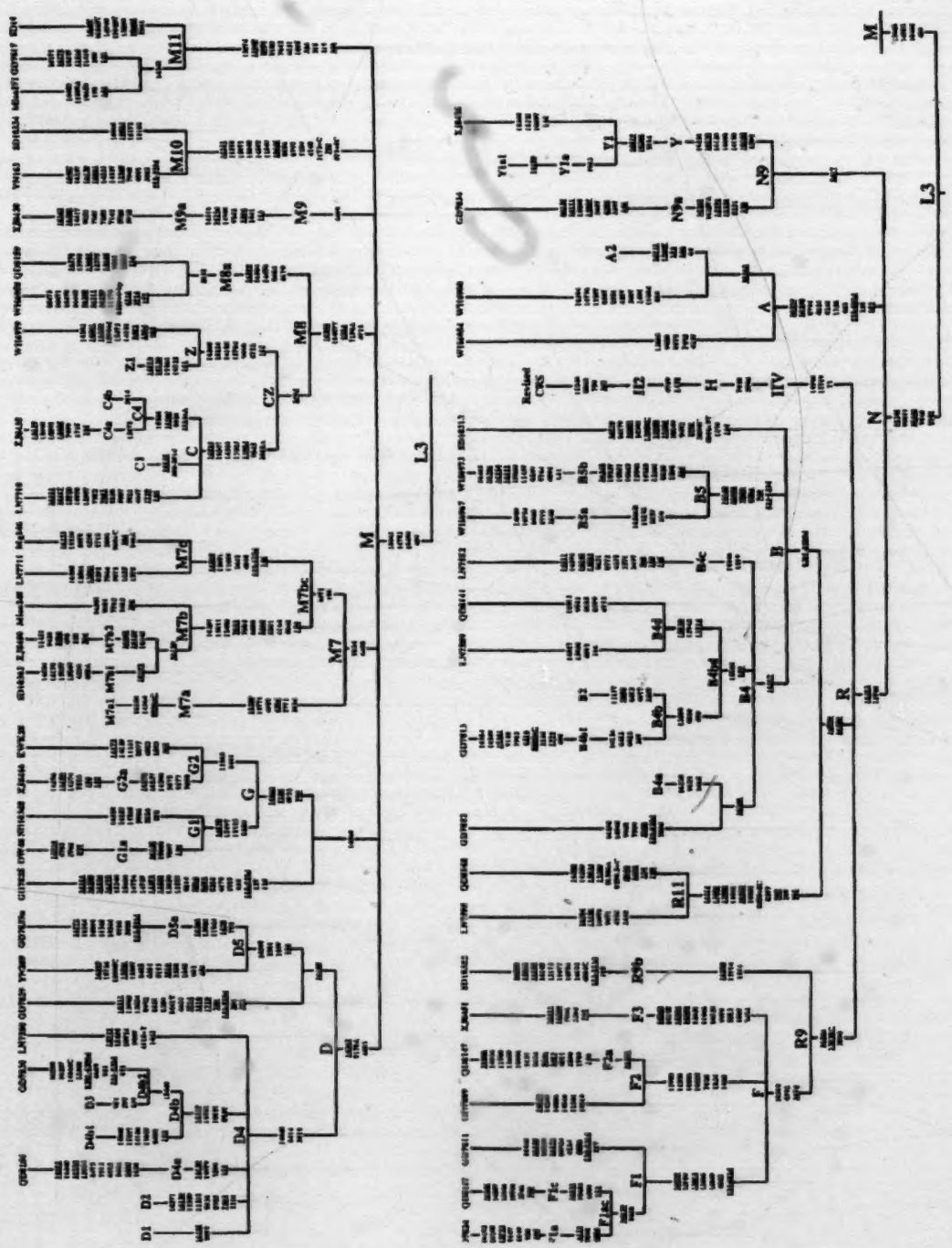


**Figure 2.5** East Asian mtDNA Phylogenetic Tree (from Kivisild *et al.*, 2002)

**Table 2.1.** Culture history model for the Lake Baikal region, Siberia (after Weber *et al.* 2002).

| Period | Culture | $^{14}$C age BP | Calibrated age BC |
|---|---|---|---|
| Late Mesolithic | Early Kitoi | ~8000–7000 | ~6800–5800 |
| Early Neolithic | Late Kitoi | ~7000–6100 | ~5800–4900 |
| Middle Neolithic | Hiatus | ~6100–5300 | ~4900–4200 |
| Late Neolithic | Early Serovo-Glazkovo | ~5300–4800/4400 | ~4200–3400/3000 |
| Bronze Age | Late Serovo-Glazkovo | ~4800/4400–3300 | ~3400/3000–1000 |

**Table 2.2** Summary of hypothesized differences between the Kitoi and Serovo-Glazkovo groups. (after Weber *et al.* 2002)

| | Kitoi | Serovo-Glazkovo |
|---|---|---|
| ***Subsistence and diet*** | | |
| Mobility | Lower | Higher |
| Annual range | Small | Large |
| Diet focus | Deer, more fish | Deer, less fish, seal |
| Resource selectivity | Narrow spectrum | Broad spectrum |
| Food supply | Erratic | Sustainable |
| Food distribution | Non-equitable | Equitable |
| ***Social relations*** | | |
| Density | Lower | Higher |
| Group isolation | High | Low |
| Sex ratio | Female-poor | Female-balanced |
| Age ratio | Child/senescent-poor | Child/senescent-rich |

**Table 2.3** Summary of archaeological and osteological data from Shamanka II individuals sampled for DNA analysis

| Master ID* | Sample ID | Cranium | Row | Morphological sex | Age (in years) | $^{14}$C age BP | Calibrated $^{14}$C age BC | Collagen Yield (%) |
|---|---|---|---|---|---|---|---|---|
| SHA_2001.011.02 | 2002.164 | present | 1 | male | 30-40 | 6860 ± 70 | 5640–5670 | 9.0 |
| SHA_2001.011.02 | 2002.165 | present | 1 | female | 18-20 | 6640 ± 70 | 5625–5510 | 1.2 |
| SHA_2002.008 | 2002.174 | present | 2 | male | 35-40 | 7020 ± 60 | 5025–4840 | 2.2 |
| SHA_2001.014.01 | 2002.178 | present | 2 | male | 25-30 | 6560 ±50 | 5550–5475 | 4.4 |
| SHA_2001.014.02 | 2002.181 | present | 2 | female | 20-25 | 6870 ± 70 | 5810–5705 | 6.2 |
| SHA_2001.019 | 2002.184 | present | 3 | male | 25-30 | 6830 ± 70 | 5750–5655 | 4.4 |
| SHA_2001.018 | 2002.186 | absent | 3 | male | 20-25 | 6790 ± 60 | 5725–5630 | 4.2 |
| SHA_2001.016 | 2002.189 | absent | 2 | undetermined | 20-25 | 6450 ± 70 | 5480–5355 | 10.3 |
| SHA_2001.013.03 | 2002.192 | present | 2 | prob. Female | 18-19 | 6890 ± 60 | 5840–5715 | 3.4 |
| SHA_1999.007 | 2002.204 | present | 1 | prob. Female | 20-25 | 6040 ± 70 | 5025–4840 | 3.2 |
| SHA_2001.015 | 2002.208 | present | 2 | male | 25-35 | 6870 ±60 | 5805–5710 | 2.1 |
| SHA_2001.012 | 2002.210 | absent | 2 | undetermined | 25-35 | 6680 ± 70 | 5805–5670 | 1.9 |
| SHA_2002.023.04 | 2002.221 | absent | 4 | undetermined | adult | 6690 ±70 | 5660–5555 | 8.6 |
| SHA_2002.023.05 | 2002.224 | absent | 4 | undetermined | adult | 6740 ± 60 | 5705–5620 | 0.6 |
| SHA_2002.023.01 | 2002.226 | present | 4 | prob. Male | 35-45 | 6340 ± 70 | 5370–5290 | 11.3 |
| SHA_2002.024.01 | 2002.229 | present | 4 | male | 25-35 | 6960 ± 70 | 5915–5740 | 10.7 |
| SHA_2002.022 | 2002.232 | present | 4 | male | 19-22 | 6110 ± 60 | 5075–4945 | 0.4 |
| SHA_2002.021.02 | 2002.235 | present | 4 | male | 25-30 | 6680 ± 100 | 5665–5515 | 1.7 |
| SHA_2002.021.01 | 2002.238 | present | 4 | male | 25-35 | 6530 ± 50 | 5520–5470 | 1.1 |
| SHA_2002.024.02 | 2002.241 | present | 4 | male | 25-30 | 6430 ± 60 | 5475–5320 | 0.7 |
| SHA_2002.021.03 | 2002.245 | present | 4 | undetermined | 16-18 | 6920 ± 60 | 5880–5725 | 3.2 |

* Master ID denotes cemetery site, followed by year of excavation, grave number, and individual number when more than one individual is present in grave.

**Table 2.4** Summary of mtDNA haplogroups at the Lokomotiv cemetery (from Mooder, 2004).

| | A | C | D | F | G2a | U5a | Other | Total |
|---|---|---|---|---|---|---|---|---|
| n | 4 | 1 | 7 | 15 | 1 | 1 | 2 | 31 |
| % | 13 | 3 | 23 | 48 | 3 | 3 | 7 | 100 |

# Chapter 3
# Approach

## 3.1. Introduction

Bone is extensively used as a source of information in archaeological research as it is generally the only human tissue preserved. Osteological features of the human skeleton are informative to physical anthropologists but because bone harbours DNA, archaeological bone samples are also of interest to molecular researchers. However, it is not uncommon for ancient bone to be altered or destroyed. For aDNA investigators, an understanding of the diagenetic processes that act upon human skeletal remains is critical, as without it, customization of laboratory protocols would be futile and authenticity of ancient human DNA research would be suspect.

Comprehension of processes and environmental factors that degrade DNA has been a significant focus of contemporary research. It is therefore necessary to review processes and factors involved in DNA decay and the limits to DNA preservation before addressing the technical issues of importance for retrieval of aDNA. This section is followed by a summary of contamination and authenticity concerns that challenge aDNA researchers. And finally, a brief outline of the techniques and protocols employed for aDNA retrieval in the Human Identification Lab for Archaeology (HILA) specifically designed for Lake Baikal samples is presented.

### 3.1.1. *Biological alteration of bone and post-mortem DNA decay*

Gilbert and colleagues (2005) state that in most cases, biological alteration in bone is caused by fungi and/or bacteria. Apparently, these modifications are not limited

to a particular burial environment. The effects of fungi and/or bacteria on bone preservation are important. They can accelerate degradation by increasing the bone porosity which simultaneously reduces chances of success for biomolecular research through loss or contamination of target molecules (Jans *et al.*, 2004).

Gilbert and others (2003) state that DNA decays rapidly after death in biological samples, and the ensuing damage is manifested in many forms (see Table 3.1). However, according to a number of aDNA researchers, two predominant classes of DNA damage exist, namely hydrolysis and oxidation.

### 3.1.2. *Hydrolysis*

Hydrolysis may be defined as the chemical breakdown of a structure through its reaction with intrinsic water. Pääbo *et al.* (2004) claim that the most obvious type of damage to DNA extracted from fossil remains is its degradation to small fragments, generally ranging in size from approximately 100 to 500 base pairs (bp). The reduction in size is due to enzymatic processes that occur shortly after death. For instance, Pääbo (1989) cites that endogenous endonuclease activity will generate strand fragmentation. As well, nonenzymatic hydrolytic attack or cleavage of phosphodiester bonds in DNA's phosphate-sugar backbone lead to the depurination of deoxyribose-adenine (A) or deoxyribose-guanine (G) bonds. These events, of course, rapidly destroy the DNA backbone (Lindahl, 1993) (Figure 3.1). The glycosidic bonds between nitrous bases and the sugar backbone are also subject to hydrolytic cleavage and result in abasic sites (Pääbo *et al.*, 2004). Once a nucleotide is released, the abasic site may undergo a chemical rearrangement that promotes occurrence of strand breakage (Pääbo *et al.*, 2004).

In addition to fragmentation and DNA modifications that hinder the extension of DNA polymerases, other known types of damage are common in aDNA. Some of these DNA modifications are problematic because although they allow the amplification of the template molecules, they cause incorrect bases to be incorporated during the polymerase chain reaction (PCR). The most common form of such modification is the hydrolytic loss of amino groups (*i.e.* deamination) from the bases adenine, cytosine, 5-methylcytosine, and guanine, resulting in hypoxanthine, uracil, thymine, and xanthine, respectively (Figure 3.1; Hofreiter *et al.*, 2001b). The deamination products of cytosine (uracil), of 5-methyl-cytosine (thymine), and of adenine (hypoxanthine) are of particular relevance for the amplification of aDNA since they cause incorrect bases (A instead of G, and C instead of T) to be inserted when new DNA strands are synthesized by a DNA polymerase (Pääbo *et al.*, 2004).

### 3.1.3. *Oxidation*

Pääbo and colleagues (2004) further assert that the length of DNA sequences that can be amplified by the PCR is limited not only by hydrolytic strand breaks, but also by lesions that present blocks to the elongation of DNA strands by the *Taq* polymerase. Many of these lesions are induced by free radicals. Examples include peroxide radicals ($O_2$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH), all of which can be created by background radiation (Table 3.1). Pääbo *et al.* (2004) point out that the major sites of oxidative attack are the double bonds of both pyrimidines and purines (Figure 3.1). The resulting damage leads to what Pääbo and others (2004) refer to as 'ring fragmentation.'

Furthermore, DNA extracted from fossil remains is susceptible to cleavage with an enzyme that acts specifically on oxidized pyrimidines, endonuclease III. Pääbo and colleagues (2004) have shown that palaeontological specimens from a diverse range of environments and ages contain these oxidized base residues. In fact, Hoss *et al.* (1996) maintain that a great proportion of aDNA is modified oxidatively via free radicals. In addition to PCR-hindering lesions, oxidative damage in DNA is most commonly seen as modification of the pyrimidines, cytosine (C) and thymine (T), to hydantoins (Pääbo, 1989), which also block the activity of PCR enzymes (Hoss *et al.*, 1996). However, a small proportion of damage events do not hinder replication but generate miscoding lesions (Pääbo, 1989). These are manifested as base modifications in the amplified sequence, changing the appearance of a DNA template and potentially generating misleading haplogroup analyses.

Gilbert and colleagues (2003) advocate that the miscoding-lesion data allow analysis of the processes involved in DNA damage and reveal a direct correlation between archaeological sites and the extent and type of damage. Their data gave no indication that sample age correlates with damage, although they found archaeological site characteristics to be a significant factor affecting DNA survival. In contrast however, Gilbert *et al.* (2003) found that in *in vitro* experiments (with a constant environment), the subsequent DNA damage did correlate with archaeological age.

Overall, oxidation, as well as the direct and indirect effects of background radiation, will modify the nitrogenous bases and the sugar-phosphate backbone of the DNA (Figure 3.1; Table 3.1). Furthermore, deamination, depurination and other

hydrolytic processes will lead to destabilization and breaks in DNA molecules. All of these processes create problems for the retrieval of aDNA sequences.

### 3.1.4. *Environmental factors: water, temperature, and soil conditions*

Smith and colleagues (2003) argue that the thermal history of a fossil is a key parameter for the survival of biomolecules. Principal factors that influence the rate of hydrolytic depurination of DNA are the burial environment's pH, the amount of chemically available water and the average temperature. Smith *et al.* (2003) assert that the first two factors are less significant in bone as bone itself exerts a substantial buffering effect between pH 4–9 and the pore size distribution of bone encourages water retention. Deep burial will buffer temperature fluctuation, but only around an annual mean, and thus temperature is likely to play a substantial role in determining DNA survival (Smith *et al.*, 2003).

Hofreiter and colleagues (2001b) emphasize that after a long enough time, the cumulative effects of damage to the DNA will become so extensive that no useful molecules remain. These researchers propose that if a burial environment's physiological salt concentrations remained constant, pH remained neutral and the temperature was maintained at 15 ºC, approximately 100,000 years would be necessary for the hydrolytic damage to destroy all DNA that could reasonably be retrieved. They add that some environmental conditions, such as lower temperatures, will extend this time limit, whereas other conditions will reduce it.

### 3.1.5. *Contamination and authentication issues*

In 1985, PCR revolutionized the ability for investigators to conduct aDNA research (Saiki *et al.*, 1985). After a decade of optimistic and fruitful study, it became apparent that the PCR and the methods employed to recover ancient human DNA were fraught with problems which have since been studied and addressed (*e.g.* Cooper and Poinar, 2000; Pääbo *et al.*, 2004). Indeed, the development of stringent criteria resulted. These procedural standards are now expected to be routinely followed when working with any ancient remains. In general, many aDNA researchers advocate that it is of paramount importance to consider and exercise *all* criteria. Pääbo *et al.* (2004) provide the most recent review of these criteria (Table 3.2).

First, a designated laboratory and equipment exclusively devoted to aDNA extraction is required. Herrmann and Hummel (1994) stress that it is crucial to complete pre-PCR and post-PCR work in physically separated laboratories. Therefore, modern DNA work in the dedicated aDNA laboratory is strictly prohibited. Cooper and Poinar (2000) suggest that it is also ideal for the pre-PCR laboratory to have an ultraviolet (UV)-filtered ventilation system and positive pressure airflow. Furthermore, they encourage the use of sterile disposables and filtered pipette tips. Bonnets, gloves, masks, booties, and lab coats/suits should be worn. As well, bleach and UV light should be used to clean and irradiate the surfaces of workbenches and equipment to destroy contaminant DNA. In particular, chemical decontamination substances, such as 30 % bleach, are used to damage and destroy surface contaminant DNA. UV irradiation is another effective and commonly employed method for decontaminating specimens, reagents and other supplies

(Yang and Watt, 2005). UV light can cause DNA to crosslink and prevent it from amplifying during PCR (Table 3.1) (O'Rourke *et al.*, 2000).

As already discussed, the great difficulty encountered in the amplification of aDNA is caused by the physical and chemical degradation of DNA templates (Hofreiter *et al.*, 2001b). Ancient DNA can only be extracted in small quantities, generally as fragments.. These fragments are usually less than 300 bp and are often associated with PCR inhibitors (Yang and Watt, 2005). Therefore, protocols for aDNA amplification must be optimized accordingly. Short DNA fragments (*i.e.* < 300 bp) should be selected as targets. Yang and Watt (2005) reinforce that the reward of selecting DNA template targets of less than 300 bp is in itself, as 'the shorter the target fragment, the greater the potential for template amplification.'

Selection of optimal DNA extraction methods (*e.g.* phenol/chloroform, silica-guanidium isothiocyanate) and setup of blanks should be carried out. Extraction blanks should be used to monitor possible contamination of extraction reagents and the entire extraction process. Experiments that involve less steps or less human involvement are considered advantageous (Yang *et al.*, 1998).

Both positive and negative controls should be incorporated with the DNA samples prepared for PCR (Yang *et al.*, 2003). Positive controls are used to indicate whether the PCR was successful and negative controls (including extraction blanks) will display amplification products if contamination occurs. O'Rourke and colleagues (2000) suggest incorporation of multiple negative controls during pre-PCR setup in order to monitor contamination more effectively. Moreover, Yang *et al.* (2003) found it preferential to include multiple quantified positive controls during their pre-PCR setup as an indicator of

the level of contamination (if it occurs), as well as the sensitivity of each PCR amplification.

Accurate assessment of the number of aDNA templates is of assistance in determining whether amplified DNA is from authentic aDNA since greater numbers of aDNA templates result in a diminished likelihood of contamination (Hofreiter *et al.*, 2001a). Competitive PCR can be used for the quantification of aDNA (Hofreiter *et al.*, 2001b), but the estimated amount of templates may also include contaminant DNA. Another proposed method to examine the preservation state of aDNA is amino acid racemization analysis (Poinar *et al.*, 1999). This type of analysis regards the change in the three-dimensional structure of amino acids from one form to a mirror image over time (Hofreiter *et al.*, 2001b). Although this analysis can detect preservation, Hoss and colleagues (1996) found that the method was not foolproof, as it may be detecting non-endogenous source(s) of DNA.

Once aDNA is amplified, it can be treated as any modern DNA sample would be; no special laboratory or equipment is required (Yang and Watt, 2005). Yang and others (2003) suggest that electrophoresis of multiple positive and negative controls should be used to examine whether contamination occurs and the level of contamination if it occurs. Sequencing results may also be of assistance in detecting contamination. For example, a DNA sample from one individual usually contains one mtDNA sequence. A good indication of possible contamination is the presence of more than one mtDNA sequence or whether the same type of mtDNA sequence is detected from many unrelated individuals.

PCR products can also be cloned to determine the number and percentage of different sequence types present in the amplified sample(s). Bower and others (2005) propose that for more ancient remains, such as fossil hominids, cloning is necessary and should be carried out. They emphasize that cloning is not only good for detecting contamination but it is also very useful in reconstructing an authentic aDNA sequence. When the number of DNA templates is extremely low and DNA itself is highly degraded, incorrect nucleotides may be incorporated into the synthesis of new DNA molecules and generate incorrect DNA sequences (Hofreiter *et al.*, 2001a), or prematurely terminated DNA fragments may jump from one template to another to produce chimeric DNA sequences (*e.g.* 'jumping PCR;' Pääbo *et al.*, 1990). These amplification errors are generally random and can be detected through cloning and repeat experiments.

Reproducibility of results is paramount to any aDNA investigation (Hofreiter *et al.*, 2001a). Some researchers propose that replication of the entire aDNA process should be undertaken by a separate laboratory to examine whether the same results can be obtained. Yang and colleagues (2003) point out that authentic aDNA and contaminant modern DNA have different behavioural patterns that should become clear when reproducibility of results is tested. If DNA is authentic, the same DNA should be extracted, amplified and sequenced from different bones of the same individuals, in different laboratories and by different groups of researchers. Thus, it should be expected that different repeats generate the same DNA sequence. However, due to its random nature, contaminant DNA generally fails in reproducibility tests (Yang *et al.*, 2003).

Overall, the underlying purpose of the aDNA authentication criteria is to examine all contamination controls, laboratory procedures and amplified DNA sequences to

demonstrate that extracted and amplified DNA is authentic aDNA from a prehistoric specimen and not contaminant modern material. Even when the outlined contamination controls are followed, contamination-free results are never guaranteed (Yang and Watt, 2005). See Table 3.3 for a list of the protocols employed in the HILA lab for rendering the ancient mtDNA results from Shamanka II samples as authentic.

## 3.2. Materials and methods for retrieval of ancient mtDNA from Siberian samples

### 3.2.1. *Samples*

Individuals excavated at the Shamanka II cemetery by Russian scholars are the subject of analysis for this thesis. Specific elements of the skeleton (*e.g.* vertebrae, ribs, cross-sections of long bone - when present) were preferentially selected and transported to Canada by BAP researchers in 2002. A total of 39 bone samples, representative of 29 individuals, were processed. Twenty-seven of these samples were prepared for aDNA extraction and subsequent amplification by previous laboratory personnel while 12 were processed by the author.

### 3.2.2. *Specimen handling and preparation*

In accordance with the strict precautionary measures outlined by Cooper and Poinar (2000) and Pääbo *et al.* (2004), all pre- and post-PCR manipulations were carried out in physically separated rooms. For this project, aDNA was extracted from human skeletal remains. Typically, the first step in preparing a bone sample for extraction of aDNA is removal of any surface contamination acquired from previous handling of the material. Yang and Watt (2005) emphasize the particular importance of this step when

dealing with human remains as they may have been handled by numerous excavators, archaeologists, and osteologists, as well as laboratory personnel. For this project, surface contamination was removed by scraping away the exposed outer layers with a sterile scalpel blade, irradiating the bone surface with ultraviolet (UV) light, followed by soaking the material in a bleach solution (*e.g.* 4 % sodium hypochlorite) (Mooder, 2004). After the surface of the bone sample was 'cleaned,' it was subjected to flash freezing in liquid nitrogen for at least two minutes in order to facilitate sample pulverization by sterile mortar and pestle. The sample was 'crushed' until a fine powder consistency was achieved. This step is critical in sample preparation, as increased surface area of the material enhances the release of the DNA during the extraction process (Boom *et al.*, 1989).

### 3.2.3. *DNA extraction*

After the sample was crushed, it was then subjected to a DNA isolation protocol first proposed by Boom *et al.* (1989), with modifications suggested by Mooder (2004). Specifically, a silica/guanidium isothiocyanate (GSN) extraction technique was employed. Yang *et al.* (1998) propose that this method of DNA purification has the advantage of being more specific for DNA and less likely to co-purify PCR inhibitors. This specificity is due to the silica particles which have a high binding capacity for DNA molecules. However, these particles are also powerful PCR inhibitors and care must be taken to ensure that the final extract is free of residual silica particles. Therefore, this procedure contrasts the traditional phenol/chloroform extraction method with the former being advantageous due to the removal of extraneous inhibitors. In fact, Mooder (2004)

customized all laboratory protocols in order to maximize both ancient mtDNA extraction success rates and yields. This type of modification is essential as the preservation of aDNA is highly dependent on local geographic conditions.

Overall, Yang and others (1998) suggest that protocols for aDNA extraction should optimize the recovery of DNA and minimize the impact of PCR inhibitors. The number of steps in the procedure should also be minimized to reduce the possibility of contamination, again, making the GSN protocol more favorable over the more involved phenol/chloroform method.

### 3.2.4. *PCR amplification*

For each sample, mtDNA extraction results were executed in duplicate in order to detect the presence of random contamination and post-mortem DNA damage. Both mtDNA extracts were then amplified by the polymerase chain reaction (PCR). Technically speaking, PCR uses a three-step process consisting of DNA denaturation, primer annealing, and DNA extension. The primers (Table 3.4) utilized target a 176 bp region of the mitochondrial hypervariable region I (HVI) from positions 16191 to 16397. This region of the genome was selected by Mooder (2004) because it contains a majority of the Asian-specific mutations in the mtDNA HVI, which are then detected using a direct DNA sequencing method. Sequencing of the mtDNA HVI is commonly employed in both ancient and modern population studies. Simply, it is the process of determining the exact order of the chemical building blocks that make up DNA. The sequencing information allows for individual haplogroup classification and thus, can reveal the maternal lineages and relationships present in the Shamanka II cemetery.

All PCR amplifications are completed on an MJ Thermocycler in 50 µL reaction volumes. Each reaction contained 1.25 U of 10X PCR Buffer (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen), 15 µg bovine serum albumin (BSA; NEB), 0.2mM of each dNTP (PE Biosystems), 200 pmol of each relevant primer, and 1.25 U of Platinum *Taq* Polymerase (Invitrogen). DNA extracts were not quantified. Instead, as outlined by Mooder (2004), 8 µL of DNA sample template was added to each PCR reaction mixture. The reaction conditions included initial denaturation at 95 °C for 2 minutes, followed by 50 cycles of 95 °C for 1 minute, 54 °C for 1 minute and 30 seconds and 72 °C for 1 minute.

### 3.2.5 *Appropriation of methods*

The methodological framework for the genetics module of the BAP was developed by Fiona Bamforth and Karen Mooder. Initially, a combined strategy of restriction fragment length polymorphism (RFLP) and hypervariable region I (HVI) sequencing of the mtDNA of ancient Siberian samples was instituted. Mooder had great success with this dual approach, as seen in her dissertation research. However, it was later decided that the RFLP approach was unnecessary. HVI sequencing alone was deemed to be more cost and time effective as well as an independently reliable means of haplogroup determination for ancient samples. However, the author was already conducting laboratory research before this protocol change occurred; therefore, the author has both RFLP and HVI sequencing results; however there is more HVI sequencing data in comparison to RFLP results.

### 3.2.6 *Restriction fragment length polymorphism*

In molecular biology, the term restriction fragment length polymorphism (RFLP) is used in two related contexts: first as a characteristic of DNA molecules (whether nuclear or mitochondrial in origin) arising from differing nucleotide sequences by which sequences may be distinguished; and second, as a laboratory technique which uses this characteristic to analyse DNA molecules. For this reason, RFLP's often serve as molecular markers for genetic assays, including genetic fingerprinting and paternity testing (Spencer, 1994).

RFLPs may result from a single point mutation in the DNA sequence which creates or deletes a restriction site or may result from inherited variable length regions in the DNA sequence (Spencer, 1994). In either case, cleavage of DNA from different individuals with restriction enzymes thus produces differing sets of restriction fragments. In correlation with the specific restriction enzyme used, such as *Alu I*, these fragments and their size allow for the classification of an individual into documented mtDNA categories (*i.e.* haplogroups).

The actual RFLP laboratory process involved the addition of a selected restriction enzyme (see Table 3.4) to the amplified ancient mtDNA product. In combination, the mixture was then incubated in a water bath at 37 °C for 16 hours. Afterwards, samples were run on a 10 % polyacrylamide gel. The gels were then stained with ethidium bromide to visualize results under ultraviolet light.

The Asian mtDNA-specific restriction enzymes and their features are provided in Table 3.5. As previously mentioned, RFLP analyses were limited by the adoption of a new analytical protocol for the ancient Siberian samples. For this reason, RFLP

classifications were obtained for only four samples. The mtDNA classification of these samples will be discussed in Chapter 4.

### 3.2.7 *Hypervariable region I of the mitochondrial genome*

A total of 29 individuals comprised the sample set for this research. All of these samples were collected in 2002 from previous excavations at Shamanka II cemetery. Of the 29, 25 consistently amplified in duplicate over an average of four occasions and were subsequently sequenced. Twenty-one of these 25 (84 %) produced authentic results which were then classified to an Asian-specific haplogroup. However, two of the 25 were deemed to be contaminant sequences, as no Asian-specific mutations were observed in these sequences. The other two of the 25 sequenced samples did not harbour enough quality DNA to produce a readable sequence. Sequencing of these particular samples was repeated but again, unsuccessfully. The remaining four undetermined individuals of the total sample set (n = 29) did not amplify consistently over repeated extractions and amplification attempts and were therefore eliminated from the examination. Sequencing results are discussed in Chapter 4.

### 3.2.8 *Amelogenin analysis*

The amelogenin locus was the target of an investigation to resolve the sex of Shamanka II samples with undetermined or ambiguous osteological sex classifications. Specifically, amelogenin refers to a gene on the X and Y chromosomes commonly used in PCR-based sex testing. The most widely used assay (see Sullivan *et al.*, 1993) amplifies part of intron 1 of the XY-homologous amelogenin gene. A single pair of PCR

primers (Table 3.6) was used to produce a product of 112 bp from the Y chromosome and 106 bp from the X chromosome. A male will therefore yield both products (double band), while a female will yield only the smaller (single band). This 6-bp difference in size is easily resolved on a polyacrylamide gel.

Following the protocol outlined by Mooder (2004), the mtDNA extracts of the 'ambiguous' Shamanka II samples were subjected to PCR amplifications consisting of 65 cycles in 50 µl reactions. The reaction mixtures were composed of 6 µl 10x PCR Buffer (Invitrogen), 20 µg BSA (NEB), 2.5 mM $MgCl_2$ (Invitrogen), 0.2 mM of each dNTP (PE Biosystems), 200 pmol of each primer (DNA Synthesis Laboratory, University of Alberta), and 2 U of Platinum Taq polymerase (Invitrogen). Like the above RFLP examinations, the amplified amelogenin products were visualised on polyacrylamide gel (12 %) and were given respective molecular sex classifications.

Eleven samples were subjected to amelogenin analysis. Nine of these eleven amplified consistently and had reproducible results over three independent trials, all of which is discussed in Chapter 4 and are shown in Table 4.4.

### 3.2.9 *Gel electrophoresis and DNA sequencing*

Following PCR, all reactions were run on 10 % polyacrylamide gels. In preparation for DNA sequencing, PCR products then underwent a modified purification step. Instead of depending on the "QuickStep 2 PCR Purification Kit" (Edge Biosystems) employed by Mooder (2004), the Qiagen "Qiaquick PCR Purification Kit" was selected due to its higher success rates relative to sample purity for samples ranging in size between 100–1000 bp. Seventy-five ng of template was sequenced for both the H

and L strands using the Big Dye terminator package (Applied Biosystems) and an ABI Avant sequencer in the Department of Medical Genetics at the University of Alberta. All sequence data were then analysed manually for the presence of substitutions deviating from the Cambridge reference sequence (Anderson *et al.*, 1981). Observation of haplogroup-specific polymorphisms allowed each sample to be classified into its respective haplogroup.

### 3.2.10 *Cloning*

All amplified mtDNA samples have been stored at -20 ºC for future cloning procedures by HILA. However, it was agreed that only ten percent of these samples need to be cloned. This percentage was accepted to be reasonable based on a review of current literature whose investigations focused on similar avenues of research. Some researchers advocate that cloning is absolutely essential for ensuring authenticity of aDNA results. Bower and colleagues (2005) contend that aDNA is a mixture of both damaged and degraded endogenous and exogenous DNA and currently, cloning is the only method that can discriminate between the mixtures and decode them into discrete samples. It was recently suggested by Bower and others (2005) that for every individual sequence of aDNA, at least 12 clones should be subsequently sequenced. Through this method, contaminant DNA can be detected and the endogenous aDNA determined by consensus.

## 3.3 Discussion and conclusion

In regards to the methodology outlined above, it is obvious that adherence to all of the authenticity criteria put forward by Paabo *et al.* (2004) was not completed with

respect to the Siberian samples. However, rigid observance to each and every suggested criterion in every case is not warranted because, as Yang and Watt (2005) note, all sources of errors do not occur in all studies.

Though amplified DNA cannot be directly determined as authentic or contaminant, it is possible to exclude one source and indirectly prove the other. Compared to the scarcity of aDNA templates, contaminant DNA is much more abundant, making contamination with modern DNA an inevitable reality in aDNA studies (Yang *et al.*, 2003). Therefore, it is necessary to analyse the possibility of contaminant DNA first before accepting a result as authentic aDNA. Negative controls, positive controls and DNA sequence analyses are all capable of indicating contamination. Each individual control itself may not have a strong power in excluding contamination, however, when all controls and analyses do not indicate contamination, there is likely no contamination (Yang *et al.*, 2003).

In addition, obtained aDNA sequences must make phylogenetic sense and/or at the very least, should not contradict genetic rules and patterns (Yang and Watt, 2005). Contamination should be suspected when opposing results occur. For example, European mtDNA should not be retrieved from the prehistoric Lake Baikal (*i.e.* 'East Asian') specimens.

Overall, there is an apparent trend in contemporary research which advocates adoption of a multidisciplinary research approach. With respect to the ancient Siberian samples analysed in this project, an integrative and collaborative approach will be undertaken. The four other research areas, or modules, that comprise the BAP provide

additional lines of evidence that may be analysed in conjunction with the ancient mtDNA dataset.

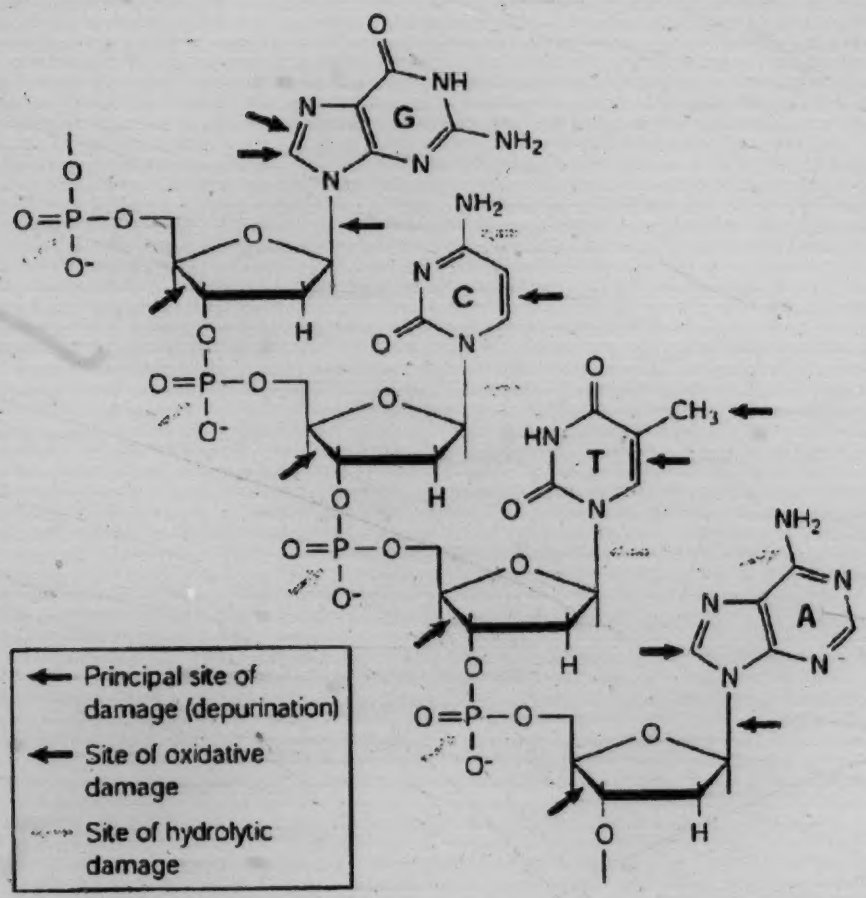


**Figure 3.1** Damage likely to affect aDNA (from Hofreiter *et al.*, 2001b). *The principal sites of damage are indicated by arrows. G = guanine; C = cytosine; T = thymine; A = adenine.*

**Table 3.1** Overview of different types of damage in aDNA as reported by Paabo *et al.* (2004)

| Type of damage | Process | Effects on DNA | Possible solutions |
|---|---|---|---|
| Strand breaks | Degradation by microorganisms | Reduction of overall DNA amounts | PCR of overlapping fragments of short length |
| | Nucleases in the postmortem cell<br>Other chemical processes | Size reduction | |
| Oxidative lesions | Damage to bases | Base fragmentation | PCR of overlapping fragments of short length |
| | Damage to deoxyribose residues | Sugar fragmention | |
| | | Nucleotide modification | Multiple independent PCRs<br>Cloning and sequencing of several clones |
| DNA crosslinks | Reactions between DNAs as well as DNA and other biomolecules | *e.g.*, Maillard products | PTB (N-phenylacyl thiazolium bromide) |
| Hydrolytic lesions | Loss of amino groups<br>1. adenine → hypoxanthine<br>2. cytosine → uracil<br>3. 5-methyl-cytosine → thymine<br>4. guanine → xanthine | Change of coding potential | Multiple independent PCRs<br>Cloning and sequencing of several clones |

**Table 3.2** Criteria of authenticity for aDNA as proposed by Paabo *et al.* (2004)

1. Cloning of amplification products and sequencing of multiple clones. This serves to detect heterogeneity in the amplification products, due to contamination, DNA damage, or jumping PCR.

2. Extraction controls and PCR controls.
Each set of extractions should include at least one extraction control that does not contain any sample material but is otherwise treated identically. Similarly, for each set of PCRs, multiple negative PCR controls should be performed to differentiate between contamination that occurs during the extraction and during the preparation of the PCR.

3. Repeated amplifications from the same or several extracts
This serves two purposes. First, it allows detection of sporadic contaminants. Second, it allows detection of consistent changes due to miscoding DNA lesions in extracts containing extreming low numbers of template molecules.

4. Quantification of the number of amplifiable DNA molecules
This shows whether consistent changes are likely to occur or not. If consistent changes can be excluded (roughly for extracts containing > 1000 template molecules), a single amplification is sufficient. Quantification has to be performed for each primer pair used as the number of amplifiable molecules varies dramatically with the length of the amplified fragment, the sensitivity of the specific primer pair used, and the base composition of the amplified fragment.

5. Inverse correlation between amplification efficiency and length of amplification
Because ancient DNA is fragmented, the amplification efficiency should be inversely correlated with the length of amplification.

6. Biochemical assays of macromolecular preservation
Poor biochemical preservation indicates that a sample is highly unlikely to contain DNA. Good biochemical preservation can support the authenticity of an ancient DNA sequence.

7. Exclusion of nuclear insertions of mtDNA
It is highly unlikely that several different primer pairs all preferentially amplify a particular nuclear insertion. Therefore, substitutions in the overlapping part of different amplification products are a warning that nuclear insertions of mtDNA may have been amplified. A lack of diversity in population studies can also be taken as an indication that nuclear insertions may have confounded the results.

8. Reproduction in a second laboratory
This serves a similar purpose as criteria 2 and 3, i.e., to detect contamination of chemicals or samples during handling in the laboratory. In our view this is not warranted in each and every study, but rather when novel or unexpected results are obtained. Note that contaminants that are already on a sample before arrival in the laboratory will be faithfully reproduced in a second laboratory.

**Table 3.3** HILA protocols for assessing authenticity of aDNA retrieved from Siberian samples (after Paabo *et al.*, 2004 and Mooder, 2004)

1. Physically separated pre- and post-PCR laboratories.

2. Dedicated aDNA lab reagents and equipment, and proper protective wear (*e.g.* bonnets, facemasks, gloves, etc. - to decrease likelihood of contaminant DNA transfer).

3. Decontamination procedures in aDNA lab (*e.g.* UV irradiation of work surfaces, equipment, reagents, and samples; bleach used on work surfaces, equipment, and samples).

4. Extraction and PCR blanks (*i.e.* 'negative controls')

5. Primers employed target short fragment (176 bp)

5. Reproducible results (*i.e.* ancient mtDNA from at least two independent extracts and more than one amplification event)

6. HVI sequencing method for determination of haplogroup polymorphism(s). Expect to retrieve ancient mtDNA results reflective of population under study (*i.e.* Asian polymorphisms)

**Table 3.4** Primers used for mtDNA amplification (from Mooder, 2004)

| Primer | Sequence | Length (bp) | Anneal Temperature |
|---|---|---|---|
| H16346 | 5'-CCCATGCTTACAAGCAAGTA | 107 | 53 °C |
| L16211 | 5'-CAGTTTAGGGAAGAGCAGGG | 176 | 53 °C |

**Table 3.5** Asian mtDNA haplogroup RFLP variation

**Asian mtDNA Haplogroup RFLP Variation**

| | | | M positive | | | M negative (N) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **M** | **M** | **C** | **D** | **E (G2a)** | **A** | **F** | **B** | **I (U5a)** | **X** |
| *Enzyme* | Alu I | Dde I | Hinc II | Alu I | Hha I/ Cfo I | Hae III | Hinc III | N/A | Hae III | Dde I |
| *Positive (+)* | cut ~80bp | cut~100bp | uncut~180bp | uncut~110bp | uncut~140bp | cut~76bp | uncut~140bp | 9bp deletion | cut | uncut~100bp |
| *Negative (-)* | uncut~123bp | uncut~120bp | cut~160bp | cut~76bp | cut~120bp | uncut~108bp | cut~90bp | no deletion (121bp) | uncut~108bp | cut~100bp |

**Table 3.6** Primers used for amelogenin amplification (from Mooder, 2004)

| Primer | Sequence | Length (bp) | Anneal Temperature |
|---|---|---|---|
| Amel 1 | 5'-CCCTGGGCTCTAAAGAAATAGTG | 112 | 72 ℃ |
| Amel 2 | 5'-ATCAGAGCTTAAACTGGGAAGCTG | 106 | 72 ℃ |

# Chapter 4
# Results and discussion

## 4.1 mtDNA polymorphisms at Shamanka II

For this section, it is important to consider that all references made with respect to Shamanka II cemetery refer to the individual human samples collected in 2002 from previous cemetery excavations. This distinction is necessary as subsequent field excavations have been completed and are ongoing. Therefore, this project must be considered a pilot analysis of the currently available data.

In total, 21 individuals excavated from 14 graves at Shamanka II were the subject of this analysis. These 21 individuals were characterized by polymorphic sites in their maternal genome, allowing for assignment to their respective mtDNA haplogroup.

### 4.1.1 *Restriction Fragment Length Polymorphism*

The Asian mtDNA-specific restriction enzymes and their features are provided in Chapter 3 (see Table 3.5). RFLP classifications were obtained for four samples. Two of these individuals were classified to haplogroup D based on a positive cut at 100 bp using restriction enzyme *Dde I* followed by an uncut band at 110 bp using enzyme *Alu I*. One individual was classified to haplogroup C based on a cut at 80 bp using the *Alu I* enzyme followed by a 180 bp fragment using enzyme *Hinc II*. Additionally, one other individual was classified to haplogroup A based on an uncut band at 120 bp using enzyme *Dde I* followed by a 76 bp fragment after treatment with enzyme *Hae III* (see Table 4.1).

### 4.1.2 *HVI sequencing results*

Sequencing results for the HVI region of the mitochondrial genome for the 2002 Shamanka II samples appear in Table 4.3. These 19 results have been combined with the RFLP results discussed above, producing a total sample size of 21 individuals successfully classified to a haplogroup. Furthermore, the RFLP haplogroup designations overlap with two of the sequenced individuals and consequently are in complete agreement. These individuals were identified as SHA_2001.011.01 and SHA_2001.015. Independent of the other, both the RFLP and sequencing data produced the same haplogroup results: SHA_2001.011.01 was classified to haplogroup C while SHA_2001.015 was classified to haplogroup A. However, two of the RFLP results stand alone as sequencing data were not obtained for these individuals. These samples include SHA_2001.014.01 and SHA_2002.023.05, both of which exhibited the characteristic RFLP features of haplogroup D (see Table 4.2). The raw mtDNA HVI sequence data are provided in the Appendix.

Of these 21 individuals, the greatest proportion (n = 7; 33 %) belonged to haplogroup D. Next, five individuals (24 %) belonged to haplogroup F. Together, these two haplogroups represent more than half of the individuals analysed from Shamanka II. Additionally, 3 (14 %) individuals belong to haplogroups A and U5a each while another 2 (10 %) were classified to haplogroup C. The one remaining individual (5 %) belongs to haplogroup G2a.

The mtDNA haplogroup distribution shows a relatively high degree of diversity for such a small sample size. However, the calibrated radiocarbon dates (from individual

human bone samples; one per individual(s) in grave) suggest a broad time scale for cemetery use, approximately 1000 years (see Table 3.5).

Various lines of evidence need to be considered alongside the mtDNA dataset in order to evaluate any patterns that should arise at Shamanka II. The multidisciplinary nature of the BAP allows for collaboration between the five modules comprising this research initiative. These complementary perspectives could include radiocarbon dating, strontium stable isotope data, demographic data, grave and burial characteristics including artifacts and associated grave goods, and spatial layout of the cemetery. However, because excavations are still in progress, these other categories of archaeological data are not available for analysis in the context of DNA data. Therefore, mtDNA results have only been considered alongside the spatial layout of the cemetery section.

**4.2 Spatial distribution of mtDNA polymorphisms at Shamanka II**

In general, most Lake Baikal region cemeteries display some type of visually identifiable spatial organization, such as rows of graves. One hypothesis to explain the relationship between graves closely associated in rows is that they represent kinship lineages. To test this hypothesis directly from a maternal perspective, mtDNA was analysed from individual human bone samples excavated from the Shamanka II cemetery. The map of Shamanka II cemetery (see Figure 4.1) depicts the spatial layout of the burials. As previously discussed, four discrete rows of closely associated graves were observed. To some extent, these rows indeed reflect the assumption that closely linked graves observed in Lake Baikal region cemetery sites manifest kinship lineages.

For instance, two separate graves occur in Row 1 at Shamanka II. One of these graves is a double burial with one individual belonging to haplogroup D. Meanwhile, the second grave contains a single individual, also determined to belong to haplogroup D. In the case of Row 2, six discrete graves were analysed. Again, the mtDNA results revealed a sharing of haplogroups, specifically A, D and U5a, by at least two individuals buried in separate graves within the row. Row 3 does not fit the lineage-based trend exemplified by the previous rows. This anomaly may be the consequence of there being only two sampled graves comprising this row. The individuals representative of the two Row 3 graves did not share the same mtDNA haplogroup. The graves of Row 4 provide the best display of maternal lineage-relationships organized by row. Four graves containing nine individuals are located within Row 4 and at least one individual interred within each of these graves is either haplogroup D or haplogroup F (see Figure 4.1). MtDNA haplogroup F is most prevalent among these Row 4 graves (n = 4; 44 %), followed by haplogroup D (n = 2; 22 %). Haplogroups A, G2a and U5a each occur once within the row. So, while Row 4 burials reveal shared mtDNA lineages between graves with respect to haplogroups D and F, this row also exhibits the greatest representation of unshared mtDNA lineages (*e.g.*, haplogroups A, G2a and U5a) of the examined sample (see Figure 4.2).

Moreover, each defined grave type, namely single, double, triple and group burials were all observed to comprise the Row 4 interments. More interesting, however, was the fact that all of the individuals buried in Row 4 were adult males. Given the evidence for sex inequality among Kitoi groups, it is parsimonious to assume that this

row was set apart from the others to distinguish these male individuals from the rest of the cemetery population.

The distribution of artifacts by grave types has yet to be comprehensively examined for Shamanka II. However, the general list of grave inclusions recovered from Kitoi graves at Shamanka II is variable and reveals no overt patterns or trends. Moreover, it has been observed that the grave inclusions recovered from Row 4 burials were not unique to the rest of the cemetery, nor were they indicative of prestige or elite status. Specifically, who was buried with what did not appear to be a factor influenced by sex of the individual and/or his/her mtDNA haplogroup affliation.

Though evidence for sex inequity has been proposed for Kitoi groups, females – in both single and double graves at Shamanka II – all possessed accompanying grave inclusions ranging in number from 14 (*e.g.*, Grave 11) to 146 (*e.g.*, Grave 14). Somewhat surprisingly, these goods included items such as lithic blanks and antler harpoons, revealing that these items were not exclusive to males. Additionally, because the cemetery only contained the remains of adults, age did not appear to be factor with respect to kind or quantity of artifacts associated with the deceased.

With the exception of G2a, all haplogroups were represented in single burials. Likewise, only haplogroups A, C, D, and F were associated with double burials. D, F, and G2a were the only haplogroups found in triple graves, while D, F, and U5a were the only haplogroups retrieved from group graves (see Figure 4.3). Therefore, haplogroups D and F occur in all grave types, with D most common in double graves and F most common in triple graves. Additionally, haplogroups A and C were only recorded in single and double burials, while haplogroup G2a is only recorded in a triple burial.

Finally, haplogroup U5a was only recovered in single and group graves. This variation is intriguing. At this stage, however, it is premature to offer an explanation. Future expansion of the Shamanka II mtDNA dataset may illuminate whether or not mtDNA haplogroup affiliation prescribed the type of grave a Neolithic Kitoi individual was buried in.

Females at Shamanka II were only recovered from single (n = 2) or double interments (n = 2). With respect to the latter case, the second individual present in the grave was always an older male. Furthermore, none of the four females represented in the study sample were classified to the same mtDNA haplogroup (see Figure 4.4). This result is interesting given that mtDNA is maternally inherited. Because the four females exhibit no maternal relationship, these preliminary findings suggest that exogamy in marriage was customary.

### 4.3. 'A tale of two Neolithic cemeteries:' Pre-hiatus Kitoi mtDNA polymorphisms from Shamanka II and Lokomotiv

The mtDNA polymorphism frequencies retrieved from the prehistoric Kitoi remains at Shamanka II cemetery mirror the previous mtDNA results obtained by Mooder (2004) from the Kitoi people buried at Lokomotiv cemetery. Haplogroup F was the most common type of mtDNA recovered from Lokomotiv, followed by haplogroup D. At Shamanka II, haplogroup D was most common, while haplogroup F trailed just behind. However, in both cases, haplogroups D and F accounted for more than 50 % of the mtDNA polymorphisms recovered from each respective population (see Table 4.4).

In order to test null hypotheses concerning associations between mtDNA polymorphism frequencies and culture group membership, exact tests of population differentiation (Excoffier *et al.*, 2005) were computed using *Arlequin* 3.01 software. The exact test of population differentiation is considered analogous to a Fisher's exact test with a two-by-two contingency table; however, the *Arlequin* 3.01 model is expanded to a table of a size defined by the number of haplogroups examined in this study (see Mooder, 2004).

Overall, an exact test approach, whether it be via population differentiation or Fisher's test, is preferable to the chi-square test for independence when dealing with small sample sizes, as the latter assumes that any given datum has a minimum frequency of five (Gould and Gould, 2002). Using an exact test approach in *Arlequin*, the mtDNA haplogroup distributions of the Kitoi at Shamanka II compared to the Kitoi at Lokomotiv observed in Table 4.6 were not found to differ significantly ($P = 0.00$).

The underlying purpose of Mooder and colleagues (2005) preliminary examination regarding the mtDNA distribution at the Lokomotiv cemetery was to understand whether the observed variability at the cemetery was used to indicate differential biological affinity (*e.g.*, mtDNA haplogroup membership) among those buried there. Their results suggested that matrilineal affinities did not overtly shape the spatial organization of the cemetery. However, they may have influenced the type of grave an individual was interred in. The most compelling differences in matrilineal affinity were found between group grave and single grave burials in one cluster of the cemetery. Mooder *et al.* (2005) state that these differences suggest an intra-community power structure that may have been shaped by matrilineally ascribed group membership.

Mooder and colleagues (2005) found haplogroup D was most prevalent among the male-dominated single graves whereas the majority of haplogroup F individuals came from group graves. This distinction led to the proposal that perhaps single graves represented the Kitoi 'elite' at Lokomotiv. At present, there is not enough data from Shamanka II to confirm or refute Mooder *et al.*'s (2005) criteria for 'elite' status among the Kitoi people at Shamanka II.

The mtDNA distribution with regard to the spatial layout of the Shamanka II cemetery has already been discussed. Analysis of this data revealed nothing of clear significance except for the all-male burials of Row 4. Generally speaking, no pattern or trend was identified. In contrast, analysis of the Lokomotiv cemetery plan revealed a spatial distinction between single and group graves. In particular, single burials were located uphill from group burials.

At Lokomotiv, 70 graves were analysed and included 43 single burials, 15 double burials, 5 triple burials and 7 multiple burials. Like Shamanka II, graves with more than one individual interred generally displayed tightly packed bodies in that these graves were not significantly wider than single graves but they were deeper. Furthermore, these group graves often revealed bodies arranged on more than one level.

Similar to Shamanka II, graves at Lokomotiv displayed the liberal use of red ochre at the burial level. Another interesting trend shared by these two cemeteries was the relatively frequent absence of crania (while the remainder of the skeleton was generally present and complete within the grave). At Shamanka II, this trend occurred 6 times out of 14 (43 %) meanwhile at Lokomotiv, it was observed 29 times out of 70 (41 %). Single burials accounted for most (four) of the missing skulls from Shamanka II

graves. The remaining two incidents were recorded from a group grave (No. 23). In contrast, at Lokomotiv, the opposite trend was detected. Only three single graves had missing skulls; the rest were missing from group burials.

Some burial characteristics observed at Lokomotiv stand out against those of Shamanka II. For instance, double graves containing male-female pairs have contrasting burial orientations. As previously discussed, male-female pairs at Shamanka II were positioned side-by-side and head-to-head within the grave. However, at Lokomotiv, male-female pairs were observed in a head-to-toe orientation within the interment. Furthermore, subadults were always recovered from group burials at Lokomotiv, while no subadult remains were retrieved from any type of grave included in this study's Shamanka II dataset.

Although the distribution of artifacts by grave types has yet to be comprehensively analysed for both Shamanka II and Lokomotiv cemeteries, it has been generally observed that grave accoutrements appear consistent across both cemeteries. Composite fishhooks and animal tooth pendants were the most common grave inclusions found at both cemeteries. Nevertheless, Mooder and colleagues (2005) report that the most abundant and diverse grave assemblages at Lokomotiv came predominantly from single burials (male and female). This result was not duplicated so far at Shamanka II. Specifically, single burials at the latter cemetery displayed enormous range in the quantity of grave inclusions in that some included up to 197 objects whereas others had no objects at all. Sex did not appear to be a significant variable at Shamanka II for determining quantity of grave inclusions as all females were recovered with associated objects.

Based on the above outlined mortuary features, namely the differential distribution of grave goods and the spatial separation of single and group graves, Mooder *et al.* (2005) proposed that single burials at the Lokomotiv cemetery may represent the Kitoi's social elite. Given that the Shamanka II mortuary data analysis is preliminary for this project and still under investigation, it is premature to extend ideas of inequality, including prestige, power and rank, to the latter. Overall, comparison of the completed mtDNA datasets (see Table 4.5) for Shamanka II and Lokomotiv revealed biological affinity between the cemeteries.

**4.4 Pre-hiatus versus post-hiatus**

Discovery of the gap in the Cis-Baikal mortuary record initially identified by analysis of radiocarbon dates inspired the formulation of the biological discontinuity hypothesis (Weber *et al.*, 2002). As shown above, the Neolithic Kitoi from Shamanka II and Lokomotiv cemeteries share the same predominant hapolgroups. Furthermore, each respective cemetery's haplogroup frequency distribution was shown to be quite similar. However, as seen below in Table 4.5, the Neolithic Kitoi share the identical haplogroups with the Bronze Age Serovo-Glazkovo recovered from Ust'-Ida I. Despite sharing the same six haplogroups, the frequency distributions between pre- and post-hiatus cemetery groups were very different. Using an exact test approach in *Arlequin*, the mtDNA haplogroup distributions of the Kitoi versus the Serovo-Glazkovo were found to differ significantly ($P = 0.00$). Thus, the general model for biological discontinuity between the pre- and post-hiatus Cis-Baikal populations is not disputed. Therefore, this outcome

independently supports Mooder's (2004) previous results indicating that the two culture groups straddling the apparent hiatus were genetically different.

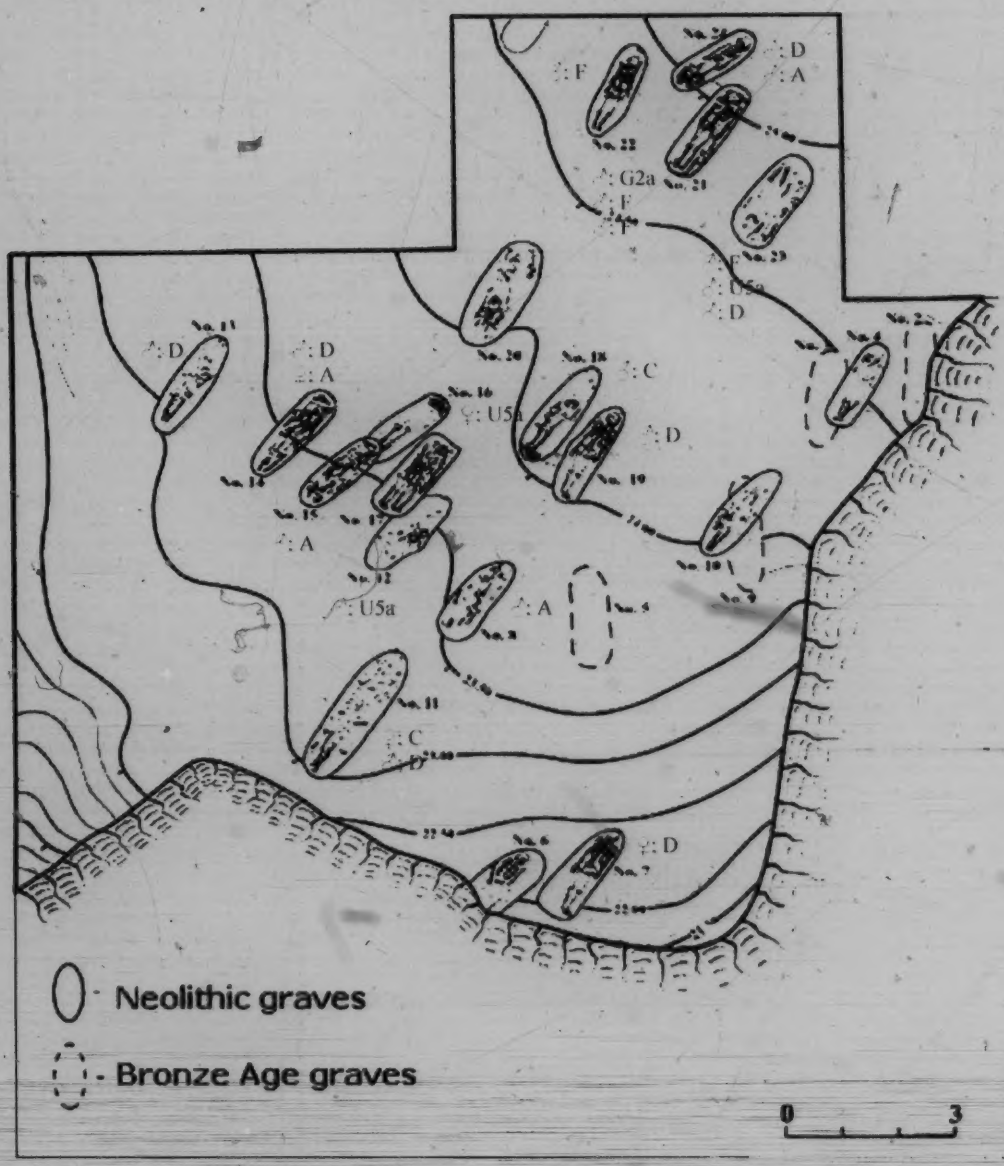


**Figure 4.1** Sex and mtDNA haplogroup distribution at Shamanka II

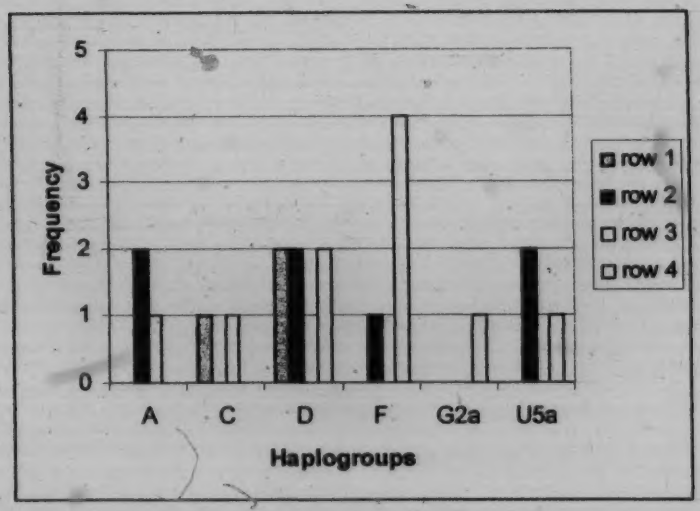


**Figure 4.2** Shamanka II mtDNA haplogroup distribution by row

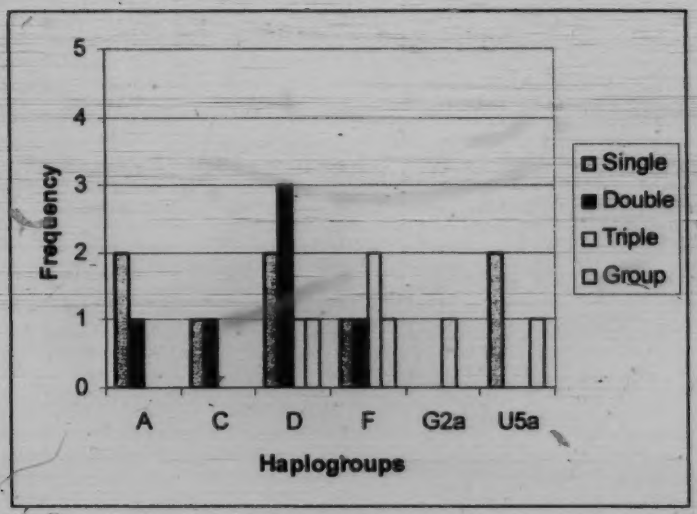


**Figure 4.3** Shamanka II mtDNA haplogroup distributions by grave type

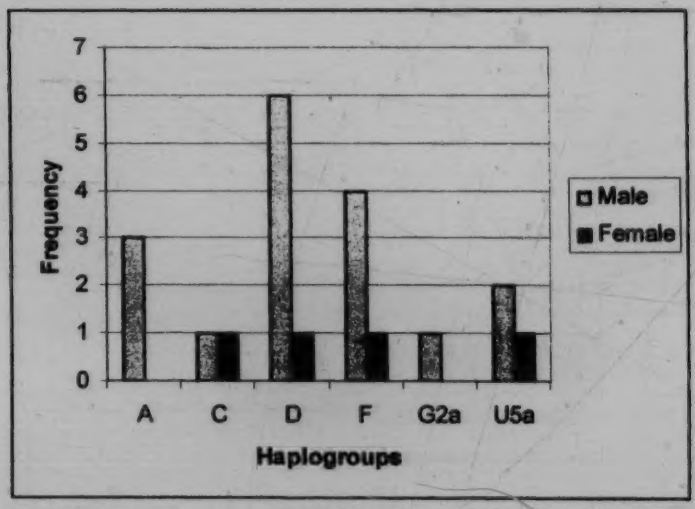


**Figure 4.4** Male and female mtDNA haplogroup frequencies at Shamanka II

**Table 4.1** Shamanka II RFLP summary

| Master ID | Sample ID | mtDNA polymorphism |
|---|---|---|
| SHA_2001.011.01 | 2002.165 | C |
| SHA_2001.014.01 | 2002.178 | D |
| SHA_2001.015 | 2002.208 | A |
| SHA_2002.023.05 | 2002.224 | D |

**Table 4.2** Summary of mtDNA haplogroups at Shamanka II cemetery

| | A | C | D | F | G2a | U5a | Other | Total |
|---|---|---|---|---|---|---|---|---|
| n | 3 | 2 | 7 | 5 | 1 | 3 | 0 | 21 |
| % | 14 | 10 | 33 | 24 | 5 | 14 | 0 | 100 |

**Table 4.3** Summary of Shamanka II mtDNA and molecular sexing data

| Master ID | Sample ID | HVI variants from CRS + 16000 | Haplogroup | Age (in years) | Morphological sex | Molecular sex |
|---|---|---|---|---|---|---|
| SHA_2001.011.02 | 2002.164 | 223 319 | D | 30-40 | male | N/A |
| SHA_2001.011.01 | 2002.165 | 223 298 327 | C | 18-20 | female | N/A |
| SHA_2002.008 | 2002.174 | 223 290 319 | A | 35-40 | male | N/A |
| SHA_2001.014.01 | 2002.178 | N/A | D | 25-30 | male | N/A |
| SHA_2001.014.02 | 2002.181 | 232 249 304 311 | F | 20-25 | female | N/A |
| SHA_2001.019 | 2002.184 | 223 319 | D | 25-30 | male | N/A |
| SHA_2001.018 | 2002.186 | 223 298 327 | C | 20-25 | male | N/A |
| SHA_2001.016 | 2002.189 | 256 270 | U5a | 20-25 | undetermined | XX |
| SHA_2001.013.03 | 2002.192 | 223 | D | 18-19 | prob. Female | XY |
| SHA_1999.007 | 2002.204 | 223 | D | 20-25 | prob. Female | Undetermined |
| SHA_2001.015 | 2002.208 | 223 290 319 | A | 25-35 | male | N/A |
| SHA_2001.012 | 2002.210 | 256 270 | U5a | 25-35 | undetermined | XY |
| SHA_2002.023.04 | 2002.221 | 256 270 | U5a | adult | undetermined | XY |
| SHA_2002.023.05 | 2002.224 | N/A | D | adult | undetermined | XY |
| SHA_2002.023.01 | 2002.226 | 232 249 304 311 | F | 35-45 | prob. Male | Undetermined |
| SHA_2002.024.01 | 2002.229 | 223 290 311 319 | D | 25-35 | male | N/A |
| SHA_2002.022 | 2002.232 | 232 249 304 311 | F | 19-22 | male | N/A |
| SHA_2002.021.02 | 2002.235 | 232 249 304 311 | F | 25-30 | male | XY |
| SHA_2002.021.01 | 2002.238 | 223 227 262 278 | G2a | 25-35 | male | N/A |
| SHA_2002.024.02 | 2002.241 | 223 290 319 | A | 25-30 | male | N/A |
| SHA_2002.021.03 | 2002.245 | 232 249 304 311 | F | 16-18 | undetermined | XY |

Note: The osteological data from excavations at Shamanka II in 2002 was completed by Dr. Angela Lieverse using standard anthropological approaches (see Lieverse, 2002).

**Table 4.4** Summary of mtDNA haplogroups at Neolithic Kitoi cemeteries

| Site | A | C | D | F | G2a | U5a | Other | Total n (%) |
|---|---|---|---|---|---|---|---|---|
| Shamanka II | 3 (14 %) | 2 (10 %) | 7 (33 %) | 5 (24 %) | 1 (5 %) | 3 (14 %) | 0 (0 %) | 21 (100) |
| Lokomotiv | 4 (13 %) | 1 (3 %) | 7 (23 %) | 15 (48 %) | 1 (3 %) | 1 (3 %) | 2 (7 %) | 31 (100) |

**Table 4.5** Summary of mtDNA haplogroups at Lake Baikal region cemeteries

| Period | A | C | D | F | G2a | U5a | Other | Total n (%) |
|---|---|---|---|---|---|---|---|---|
| Neolithic | 7 (13 %) | 3 (6 %) | 14 (27 %) | 20 (38 %) | 2 (4 %) | 4 (8 %) | 2 (4 %) | 52 (100) |
| Bronze Age | 10 (26 %) | 11 (28 %) | 2 (5 %) | 3 (8 %) | 4 (10 %) | 0 (0 %) | 9 (23 %) | 39 (100) |

## Chapter 5
## Conclusion and future research

Pääbo and colleagues (2004) propose that the study of ancient DNA has "the allure of time travel." Indeed, an investigation where authentic ancient DNA (aDNA) is successfully retrieved from human bone samples allows researchers to analyse aspects of the biological history of prehistoric individuals and populations. When complementary evidence is available, such as archaeological data, potential for reconstruction of past life ways and underlying forces involved in the apparent 'shift' of cultures is enhanced.

### 5.1 Summary

In order to address the objectives of this project, background information regarding Lake Baikal region geography, archaeology and culture history was provided. This section was followed by a review of mtDNA and its unique features, as well as a comprehensive literature summary. Next, the materials and methods employed for retrieving mtDNA from prehistoric human skeletal remains was presented. Underlying protocols and principles for achieving the greatest possibility of sample authenticity was also included.

This study reports the successful retrieval of authentic mtDNA polymorphism data from 21 individuals whose calibrated radiocarbon dates range from ca. 5900–4800 years BC. The principal objective of this study was to compare the mtDNA polymorphisms observed at Shamanka II cemetery to the mtDNA results from Lokomotiv and Ust'-Ida I cemeteries. All three cemetery populations were comprised of mtDNA haplogroups of Asian origin. More specifically, RFLP and HVI sequencing data revealed the polymorphic characteristics of haplogroups A, C, D, F, G2a, and U5a. Pre-

hiatus Kitoi and post-hiatus Serovo-Glazkovo shared all of the above haplogroups; however, the frequency distribution of each particular haplogroup differed significantly between the two culture groups.

The preliminary mtDNA results obtained from individuals at Shamanka II did not appear to be spatially organized to overtly reflect matrilineal relationships. The mtDNA results from Lokomotiv cemetery also revealed that apparent grave clusters were not organized to reflect matrilineal ties. Nevertheless, mtDNA from Shamanka II suggests that certain maternal lineages, or haplogroups consistently used this cemetery to bury their relatives over a period of approximately 1000 years.

Males and females were not equally represented among the examined samples at Shamanka II. In fact, a 4 to 1 ratio of male to female burials was found. Additionally, each female (n = 4) sampled at Shamanka II was determined to belong to different mtDNA haplogroup. These two features are intriguing and merit continued investigation. Exogamy in marriage practices is implicated through these early results from Shamanka II.

The basic rules of marriage are those of exogamy and endogamy. The rules of exogamy essentially indicate which relatives are forbidden to marry. Of course, this practice is complicated by a population's perceived notion of how individuals are related (*e.g.* consanguineously, affinally, fictively, *etc.*). The rules of endogamy generally require that a marriage be exclusively or preferentially contracted within specific social groups, or among particular relatives, such as cross cousins.

Matrilocal post-marital residence is determined by a societal rule that requires a woman to remain in her mother's household after marriage. Her husband must leave his

family and move in with her. The consequences of this practice within a population using the same cemetery over multiple generations would leave an obvious genetic signature within the mortuary record. A molecular archaeologist would expect to find a very consistent pattern of maternally inherited mtDNA from both the men and women of the original population. Theoretically, 'foreign' men introduced into the population through marriage should possess different mtDNA, as well as different Y chromosome DNA, creating a prominent distinction between them and the remainder of the mortuary population under examination.

Patrilocal post-marital residence directly contrasts the matrilocal rule. In this case, the societal rule sees that a man remains in his father's household after marriage, while his wife must leave her family to move in with him. In a population practicing this post-marital custom, one would expect to find consistency in Y chromosome DNA in the males of the original population. Theoretically, women introduced into the population through marriage should be recognized by disparate mtDNA.

Overall, analyses of both matrilineal and patrilineal DNA markers are necessary to characterize post-marital associations. With more information, the intent is to explore if it is possible to determine marriage practices and post-marital residence patterns for both the pre- and post-hiatus groups.

Subtle differences in mortuary customs were noted between the Neolithic Lokomotiv and Shamanka II cemeteries (Chapter 4). This variance only emphasizes the social complexity of prehistoric hunter-gatherer groups in that they may be classified to the 'same' cultural group (based on mortuary protocol and biological/genetic affinity), but sociocultural customs, especially those that govern mortuary ritual are not static

between time and space. These unique and defining features of Lake Baikal region cemeteries add to the intrigue of funereal sites in general and warrant their continued research.

Overall, the results of this project produce a small snapshot of a much larger picture that is currently incomplete. Excavation and analysis of Shamanka II materials are currently ongoing and therefore it is anticipated that with continued work, the mortuary customs and biological relationships among the Neolithic Kitoi will be enhanced.

**5.2 Future research**

Adopting a more comprehensive genetic approach for future analyses holds tremendous potential for revealing more information regarding the mortuary practices of pre- and post-hiatus Cis-Baikal cemetery populations. Including ancient Y-chromosome DNA analysis to the BAP human genetics module should enhance current understanding of prehistoric Cis-Baikal mortuary behaviour, which to date, has been based primarily on mtDNA data. A paternal-inheritance perspective could reveal key evidence with respect to social organization and mortuary custom, especially if these group features are patrilineally-based, as is suggested from the male-dominated sex ratios at pre-hiatus cemeteries.

Genetic analyses of prehistoric human skeletal remains may also be enhanced by investigating intra-cemetery biological relatedness with autosomal short tandem repeat (STRs) molecular markers. An autosomal DNA perspective is much the same as forensic methods where individual identification is the ultimate goal of genetic investigation.

Specifically, STRs are regions within an individual's nuclear DNA that contain specific DNA sequences tandomly repeated a number of times (Spencer, 2004). STRs consist of smaller repeat units (2–7 base pairs long) repeated fewer times (7–40 repeats per STR region). The short lengths of STR regions increase the potential of successful amplification via PCR and allow this method of analysis to be applied to ancient samples whose DNA is expected to be damaged and/or degraded (Spencer, 2004). Whereas mtDNA and Y chromosome DNA only reveal maternal and paternal relationships, respectively, the power of autosomal STRs lies in their ability to discern familial relationships, including parents and offspring. Specifically, STR analyses would allow for a more precise assessment of parent-offspring relationships within a cemetery population. Therefore, when aDNA is preserved well enough that nuclear DNA may be retrieved, autosomal STR markers would be an ideal method for determination of individual identity and biological kinship reconstruction with respect to mortuary populations. When united with Y chromosome, mtDNA, and all other mortuary archaeological data (including spatial data of cemetery), it becomes increasingly possible to reconstruct a comprehensive picture of the organization of funereal sites, as well as the biological population affinities between the analysed cemetery population and its ancient and modern analogs.

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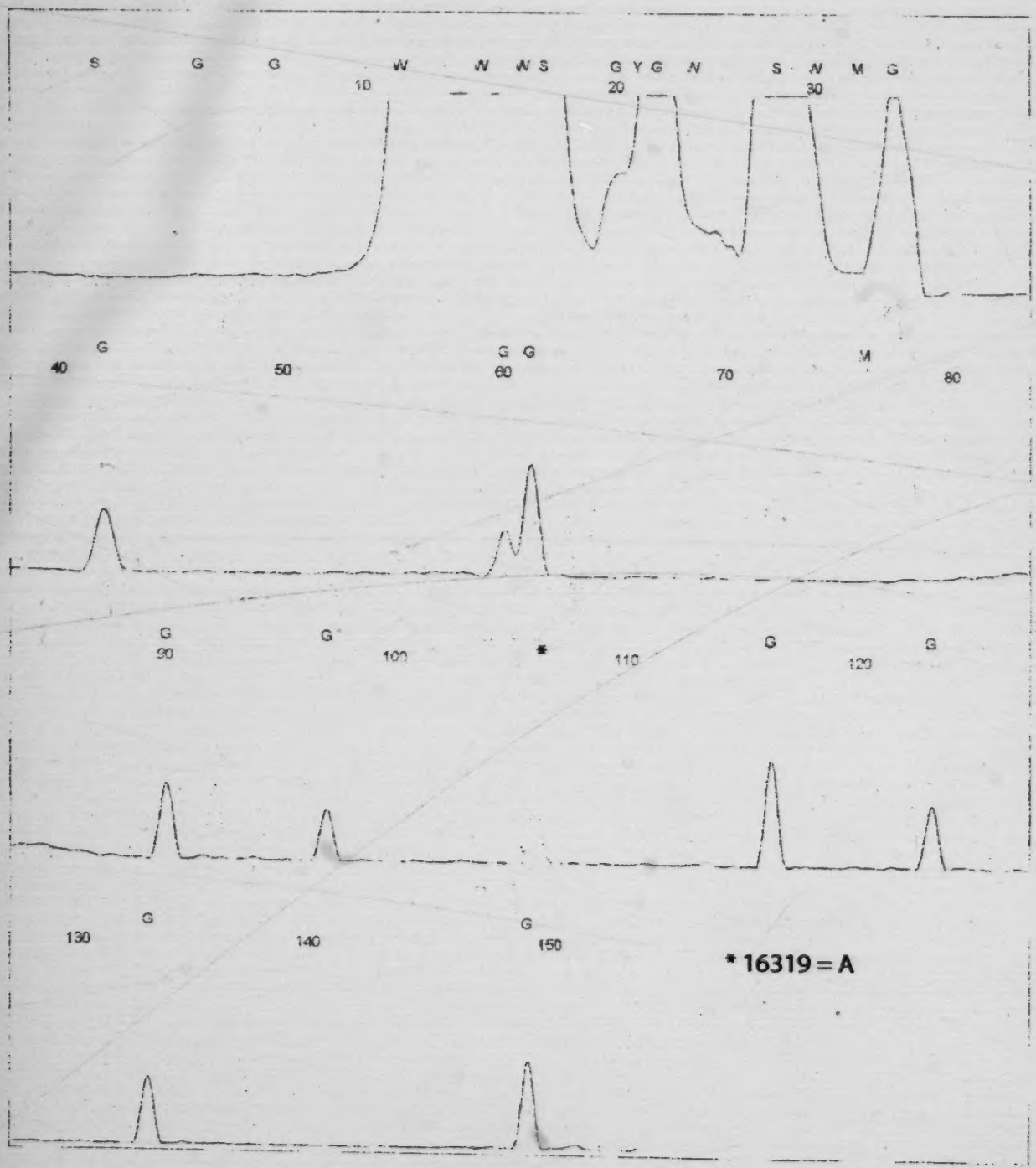
## Appendix
## Representative samples of aDNA HVI sequences

## SHA_2002.008 (2002.174): Haplogroup A, H-strand

**File: 174H**

www.geospiza.com

Sample Name: 174H
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 14.3898
Comment: TIA



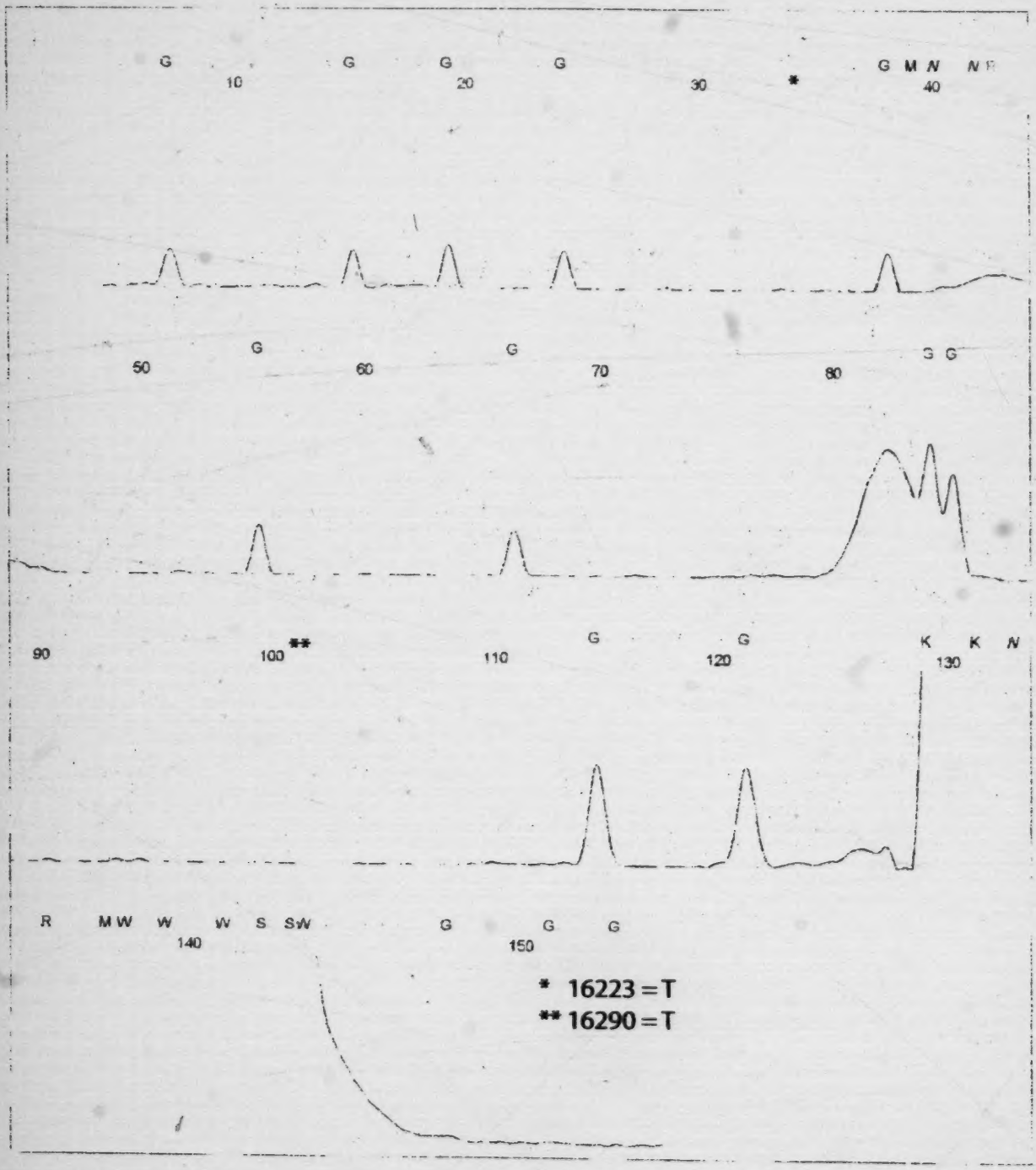

FinchTV v.1.3.1

## SHA_2002.008 (2002.174): Haplogroup A, L-strand

**File: 174L**

www.geospiza.com

Sample Name: 174L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 14.3044
Comment: TIA

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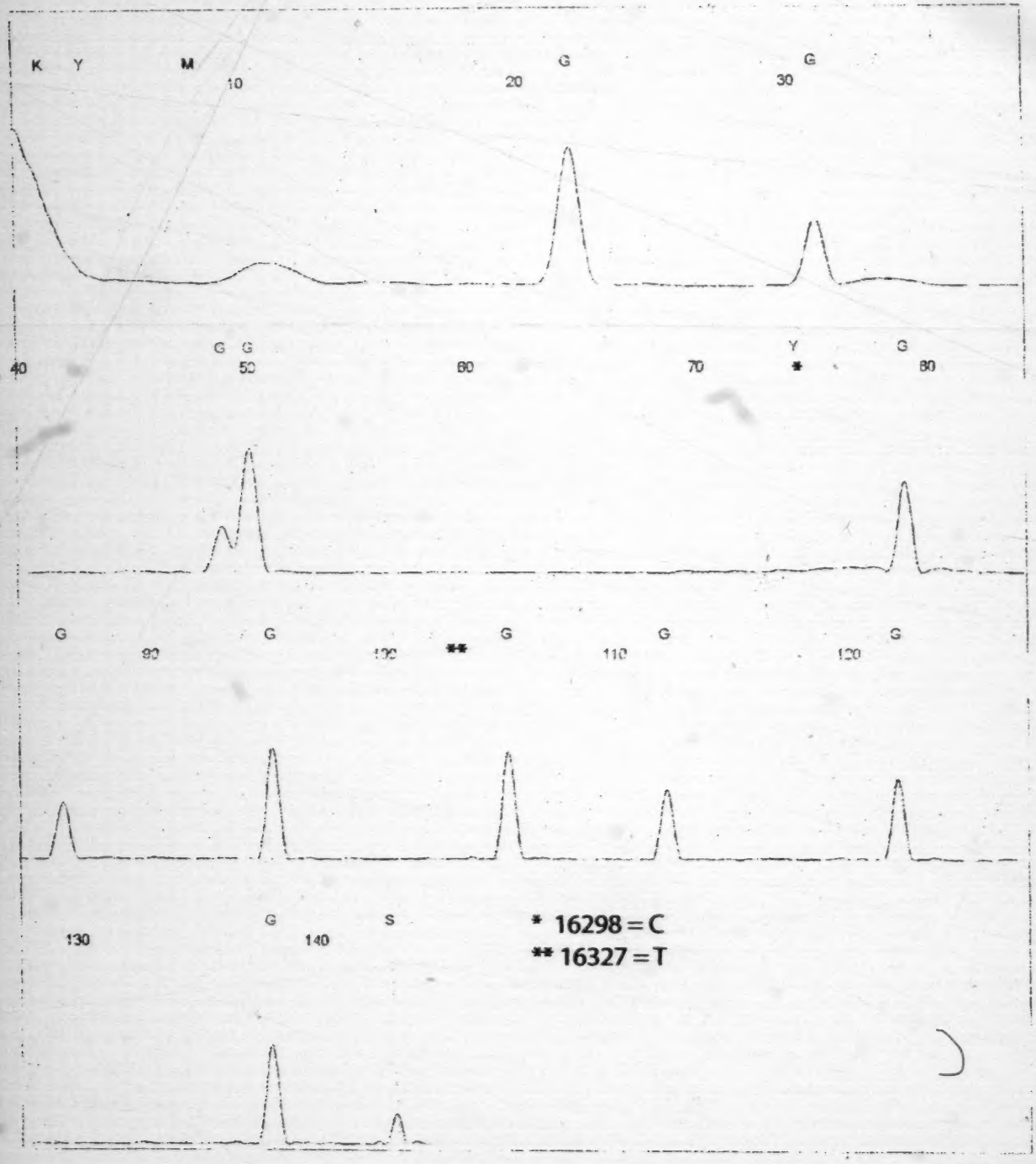
## SHA_2001.011.02 (2002.165): Haplogroup C, H-strand

**File: 165H**

www.geospiza.com

Sample Name: 165H
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 13.2948
Comment: TIA



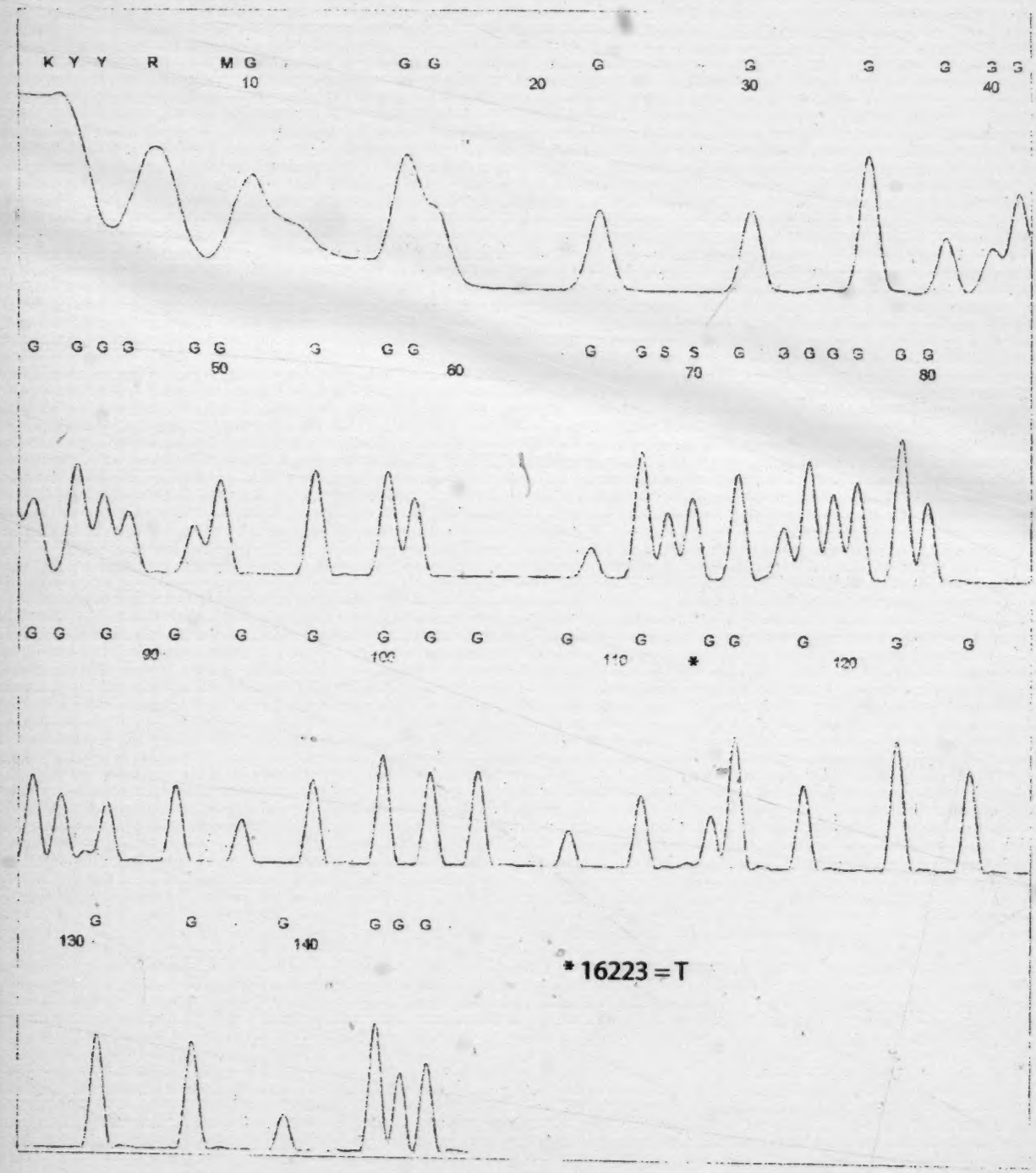

FinchTV v.1.3.1

## SHA_2001.011.02 (2002.165): Haplogroup C, L-strand

**File: 165L**

www.geospiza.com

Sample Name: 165L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 13.1873
Comment: TIA



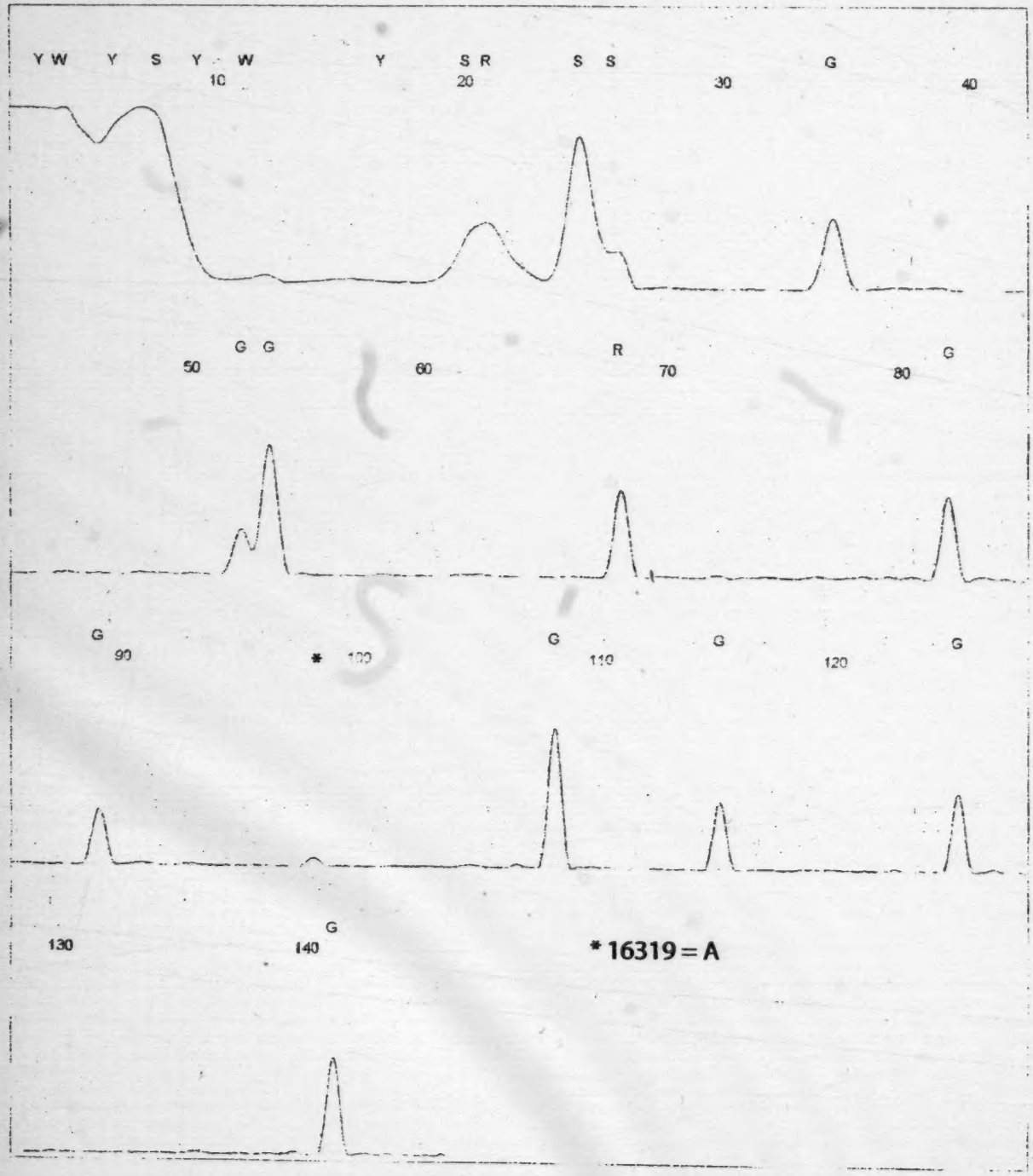

FinchTV v.1.3.1

## SHA_2001.011.02 (2002.164): Haplogroup D variant 1, H-strand

**File: 164H**

www.geospiza.com

Sample Name: 164H
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 13.4335
Comment: TIA

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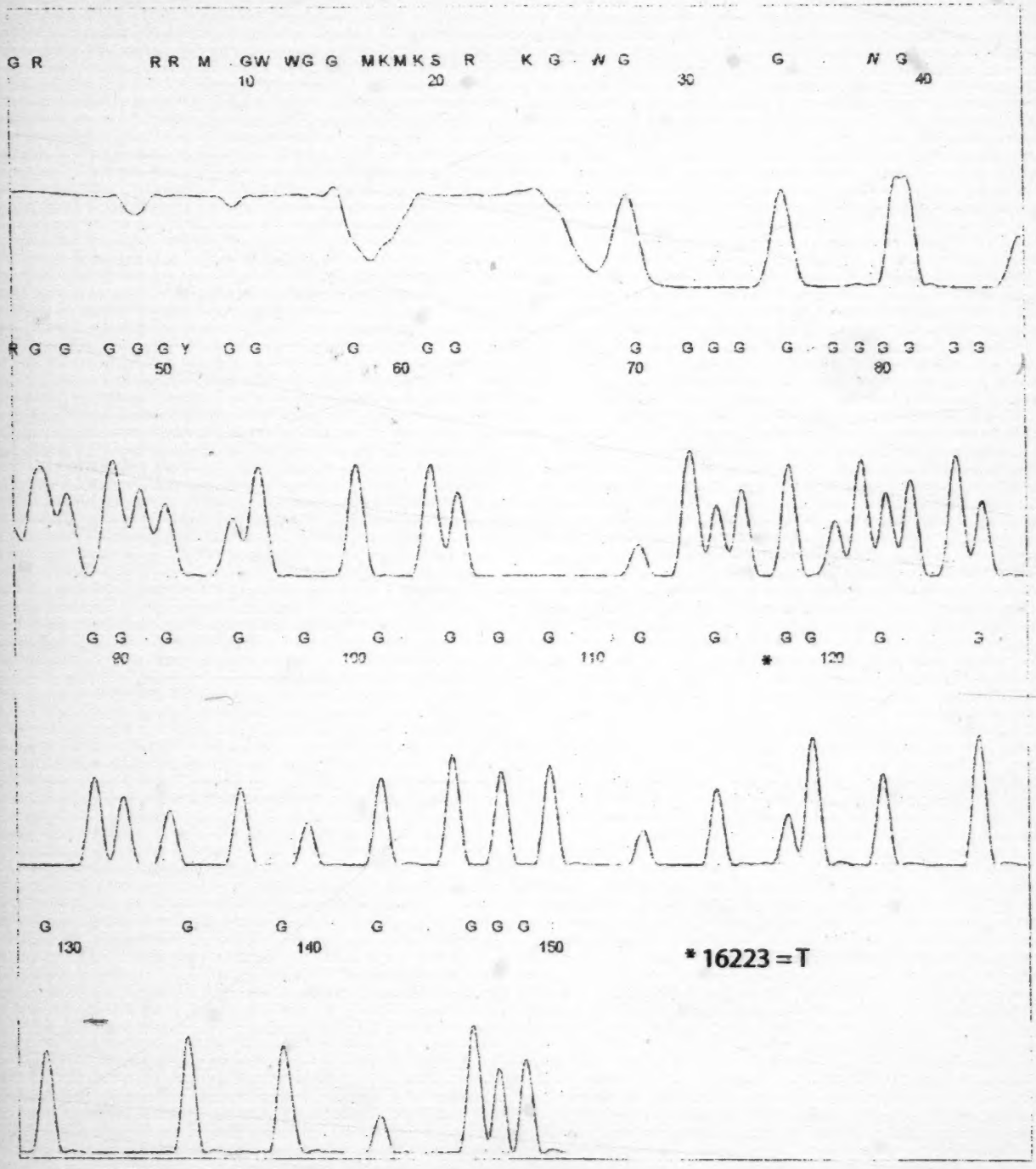
## SHA_2001.011.02 (2002.164): Haplogroup D variant 1, L-strand

**File: 164L**

www.geospiza.com

Sample Name: 164L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 13.1964
Comment: T1A

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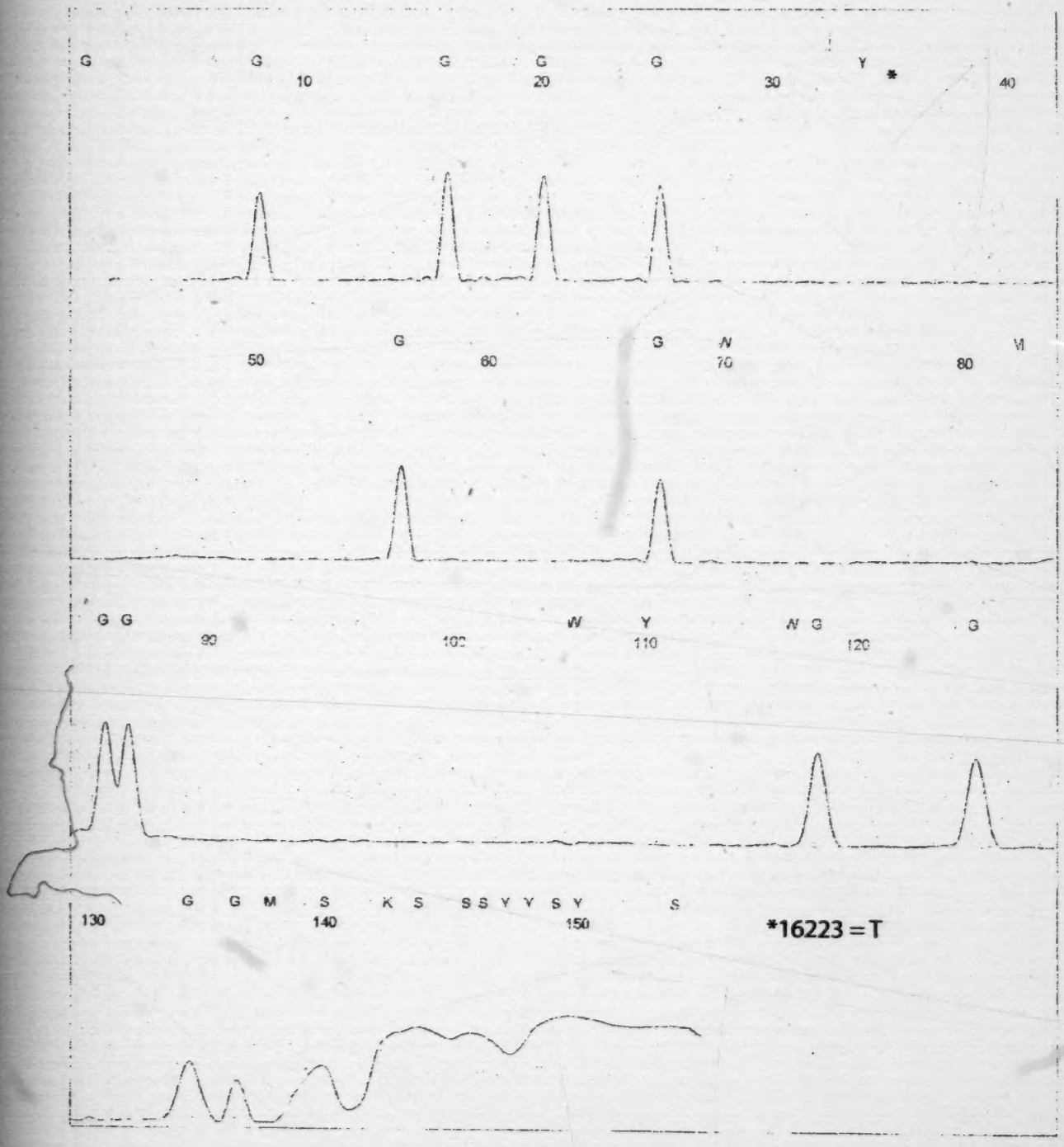
## SHA_2001.013.03 (2002.192): Haplogroup D variant 2, L-strand

**File: 192L**

www.geospiza.com

Sample Name: 192L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 14.7606
Comment: TIA

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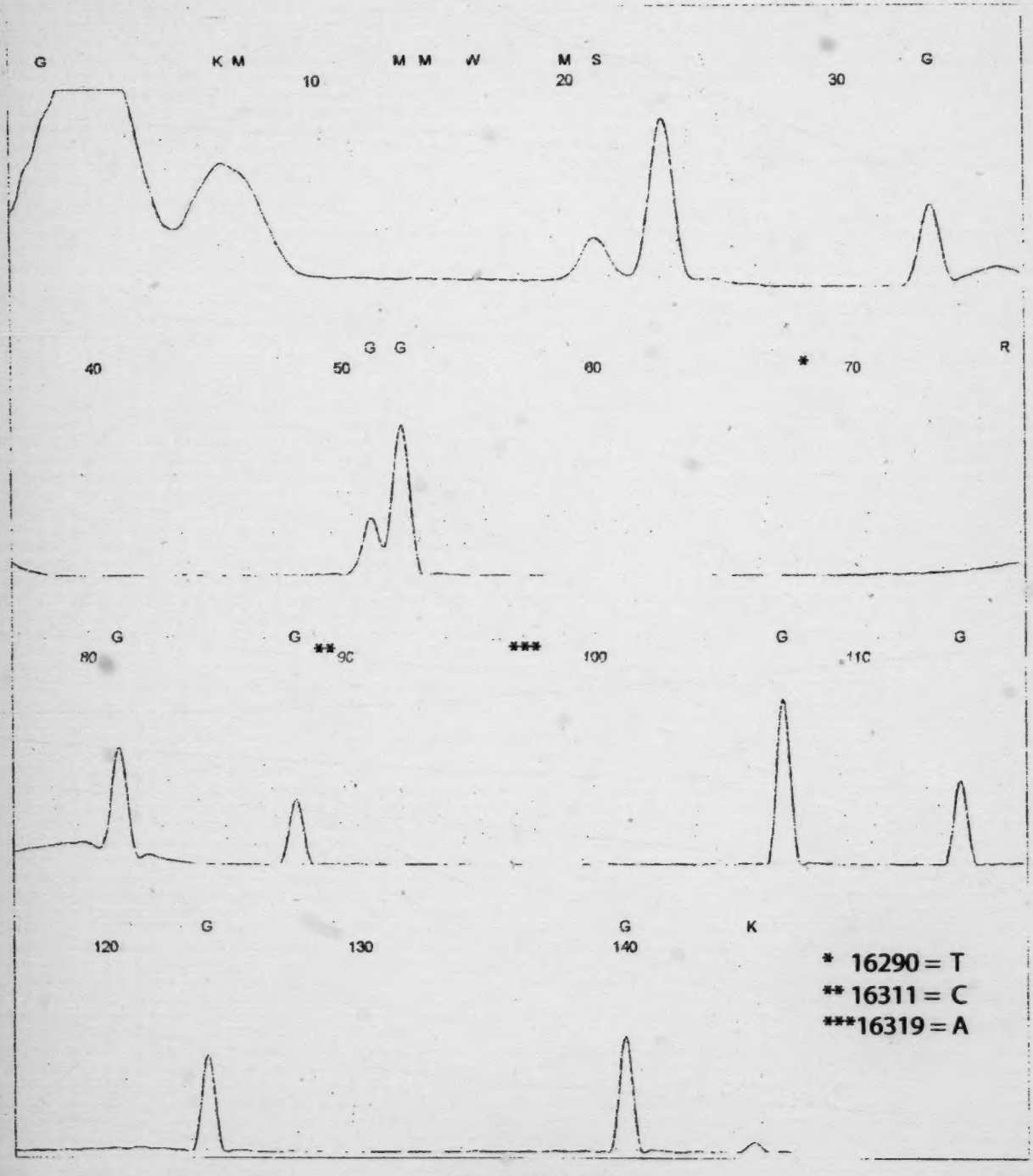
## SHA_2002.024.01 (2002.229): Haplogroup D variant 3, H-strand

**File: 229H**

www.geospiza.com

Sample Name: 229H
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 13.2326
Comment: TIA




FinchTV v.1.4.0

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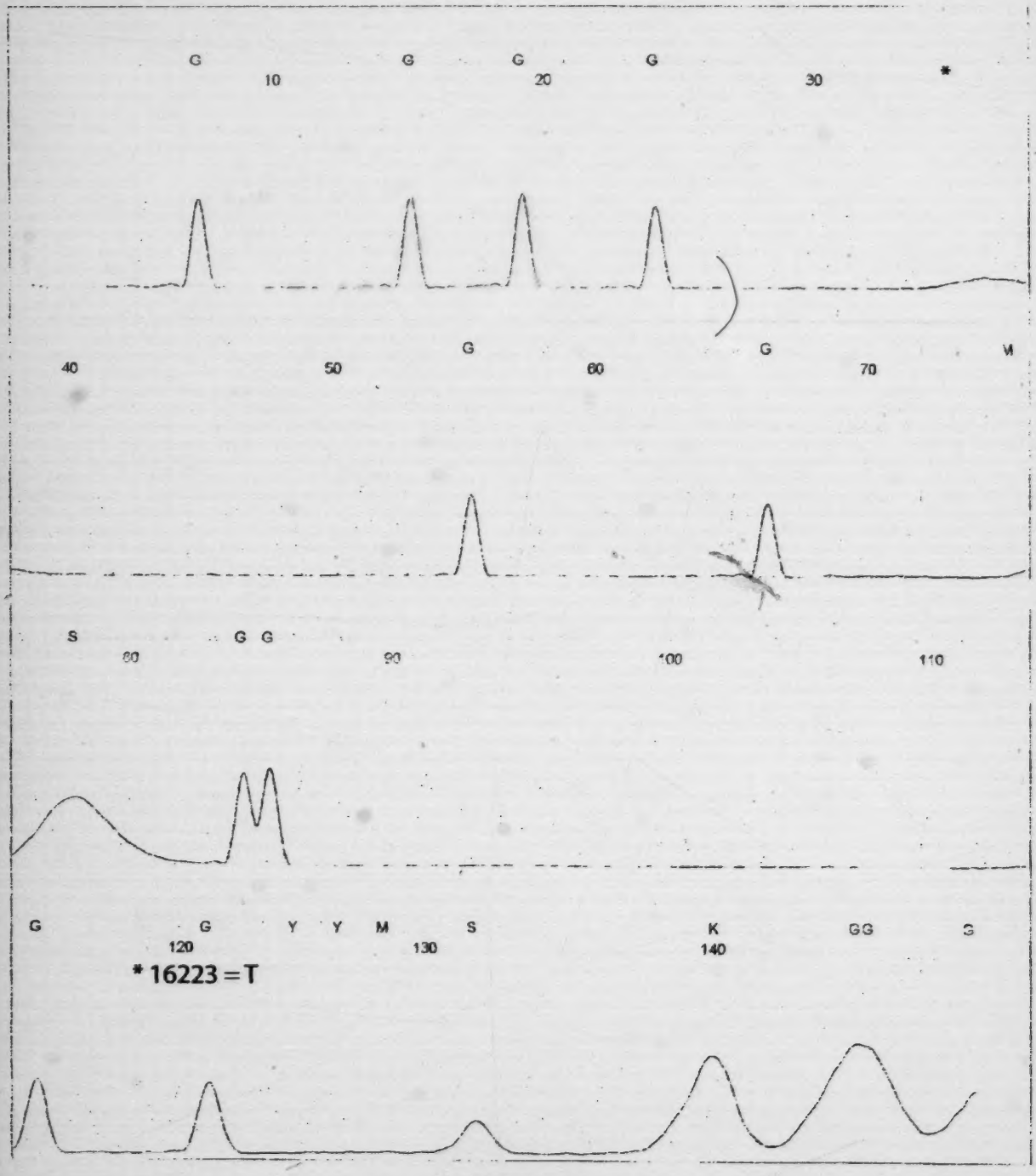
## SHA_2002.024.01 (2002.229): Haplogroup D variant 3, L-strand

**File: 229L**

www.geospiza.com

Sample Name: 229L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 13.243
Comment: TIA



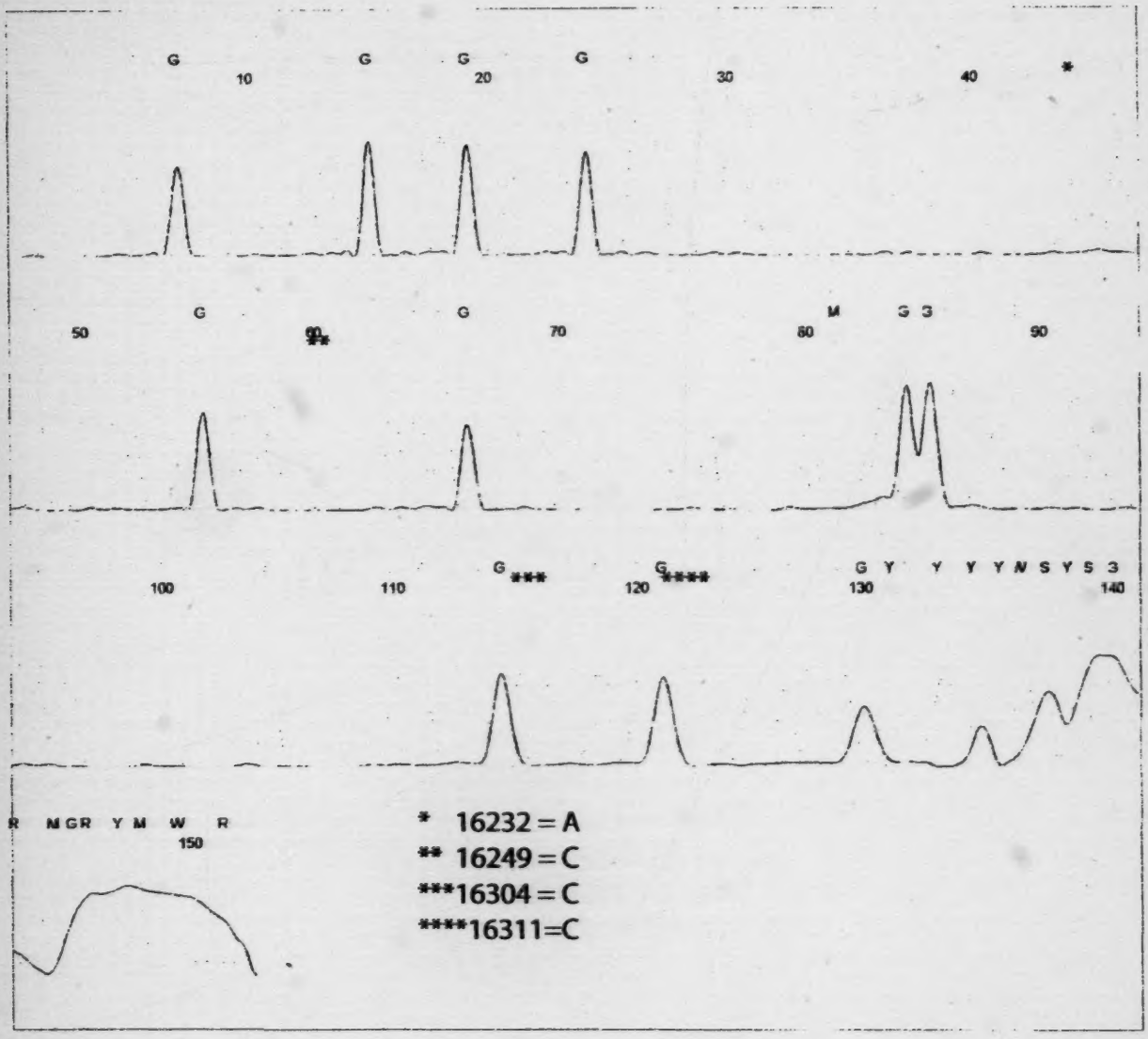

FinchTV v.1.4.0

## SHA_2002.021.03 (2002.245): Haplogroup F, L-strand

**File: 245L**

www.geospiza.com

Sample Name: 245L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 14.7191
Comment: TIA

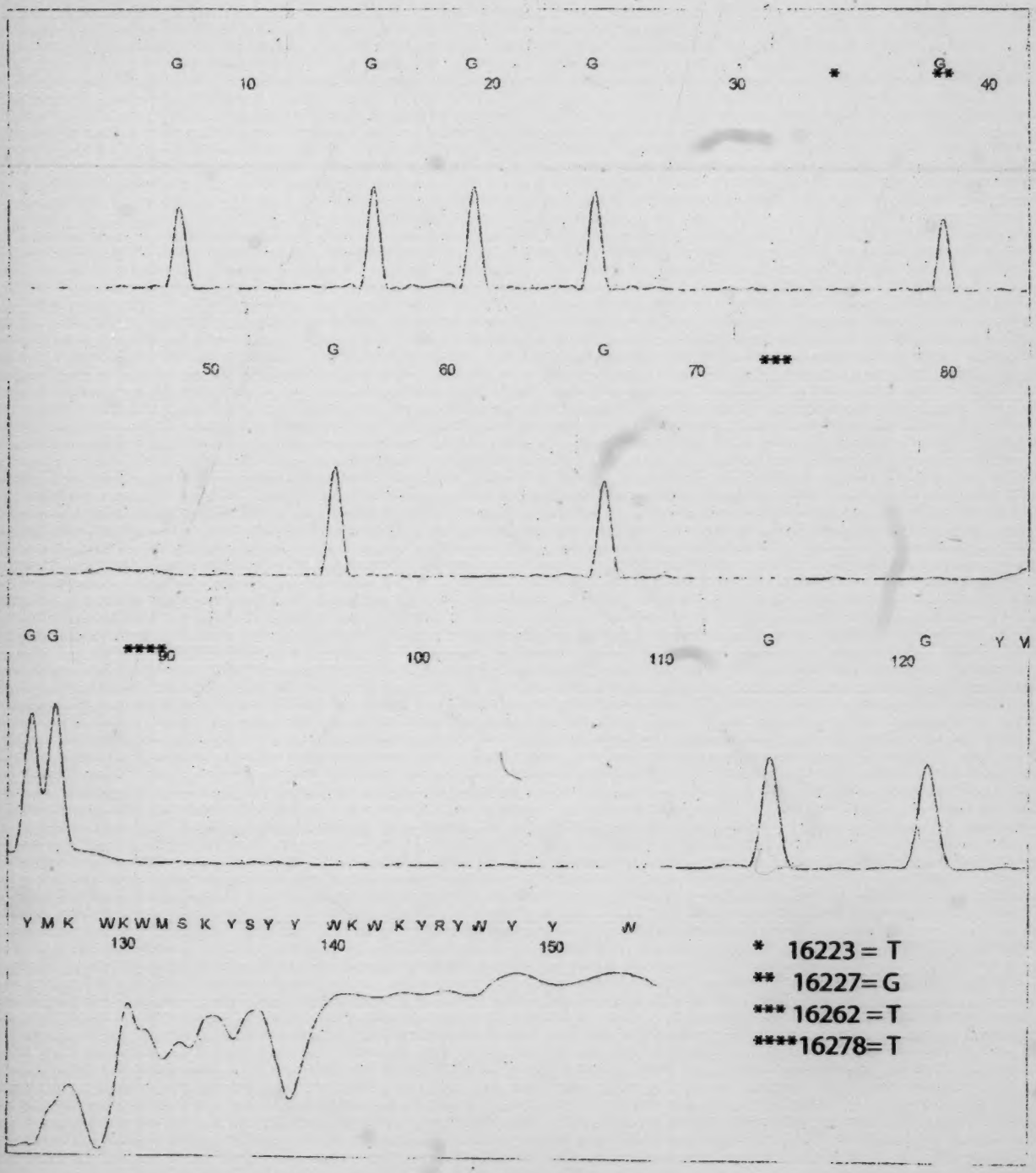


## SHA_2002.021.01 (2002.238): Haplogroup G2a, L-strand

**File: 238L**

www.geospiza.com

Sample Name: 238L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 14.9199
Comment: T1A



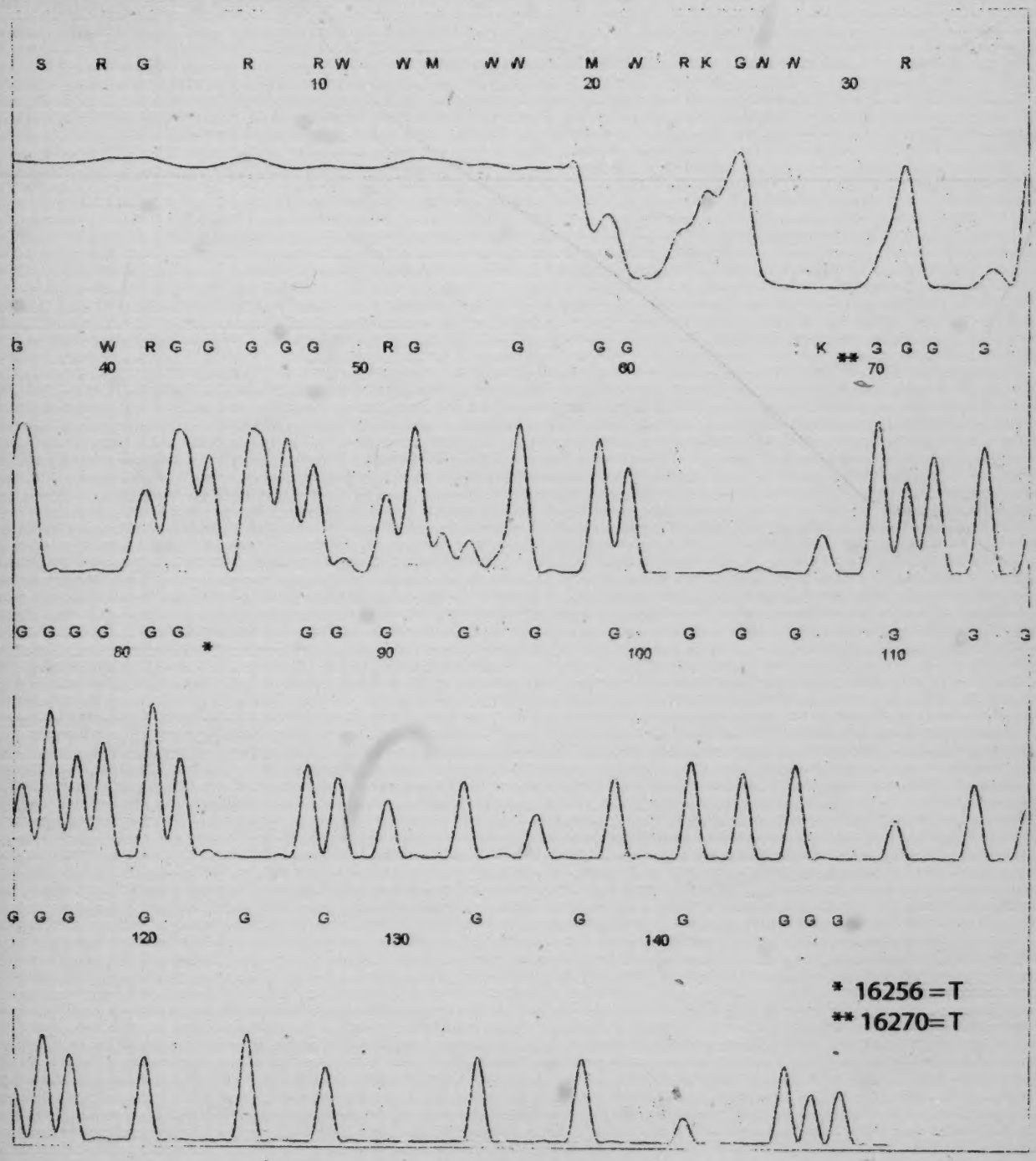

FinchTV v.1.3.1

## SHA_2001.016 (2002.189): Haplogroup U5a, L-strand

**File: 189L**

www.geospiza.com

Sample Name: 189L
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 14.9974
Comment: TIA




FinchTV v.1.4.0